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PATHOGENIC MICROÖRGANISMS

A PRACTICAL MANUAL FOR STUDENTS, PHYSICIANS AND HEALTH OFFICERS

BY

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PREFACE TO THE NINTH EDITION.

THE first edition of this book was called Bacteriology in Medicine and Surgery. It was written to make available for others the practical knowledge which had been acquired in the work of the bacteriological laboratories of the city of New York, and was intended more for medical practitioners than for medical students or laboratory workers. When the second edition had been exhausted the improvement in methods of cultivating and studying the protozoa had reached a point rendering it advantageous to include the animal as well as the vegetable germs. This was done and the title of the third edition was altered to conform with the text, which had been broadened to give in outline practically the whole field of pathogenic microörganisms. At the same time the subjects were treated in a more comprehensive manner, so as to make it a suitable text-book for medical students.

In the fifth edition the material was rearranged in order to bring together more closely all of the pathogenic organisms. Under this arrangement Part I dealt with the general characteristics and methods of study of all the microörganisms considered (bacteria, moulds, yeast and protozoa), Part II included the study of the individual pathogenic microörganisms and their near relatives and Part III presented certain practical aspects of the subject, under the title Applied Microbiology. In the sixth edition the practical application of serums and vaccines was transferred also to Part III. This change seems to have been of doubtful value and in this edition we have returned to the earlier arrangement. The nine plates included in this edition were arranged and drawn especially for this book.

In the eighth edition further rearrangements were made so that the grouping of different microörganisms conformed more closely to the classification adopted by the Society of American Bacteriologists. The new terminology suggested by this Society was added to the older common names and several new comprehensive tabulations were given.

In this edition, again the many additions to our knowledge during the past three years has necessitated a very extensive revision and many parts of the book have been completely rewritten.

The sections on immunity have been amplified. Our experiences to date with active Immunization against diphtheria and scarlet fever are given. Not only are the immediate practical results emphasized but also those observations which bear on the general problems of immunity. Work in this laboratory as well as work coöperatively with

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other laboratories, made possible through the interest and financial help of the Metropolitan Life Insurance Company and private individuals, has given us considerable further knowledge as to the probable value of certain serums and vaccines. These results also have been added. The chapters on the Pyogenic Cocci, on the Paratyphoid Bacilli, and the pneumococci have been practically rewritten.

The recent additions to our knowedge of scarlet fever, measles, yellow fever, undulant fever, tularemia, etc., have received due attention.

Miss Georgia Cooper revised the portion of the chapter on pneumococci dealing with the new types.

Dr. Camille Kereszturi has written the portion on the feeding of infants with the Calmette culture of tubercle bacilli.

The chapter on Complement Fixation has been revised by Miss Lucy Mishulow and Mr. John Koopman. The details regarding the newer precipitin tests, especially those of Kahn, Kline and Meinike are added.

The bibliography has been added to, special effort being made to give those references which cover the essential advances during the last few years.

The comprehensive summary table has been continued and revised, since many have expressed appreciation of it. This table gives the essential characters of and pathological conditions caused by each of the commoner microörganisms. Our teaching experience has suggested the need of the student for such means of orientation.

For assistance in proof reading and for preparation of the index we are indebted to Miss Annabel Walter and Miss Barbara Johnson.

The coöperation of all our associates in the laboratory is gratefully acknowledged. The value of the viewpoint and highly specialized experience of many individual workers cannot be too strongly emphasized.

W. H. P.
A. W. W.
C. K.

New York.

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PATHOGENIC MICROÖRGANISMS.

PART I.

PRINCIPLES OF MICROBIOLOGY.

CHAPTER I.

INTRODUCTORY HISTORICAL SKETCH.

Although most of the more important discoveries in microbiology, which place it on the footing of a science, are of comparatively recent date, the foundations of its study were laid nearly three centuries ago. From that time the history of microörganisms has been intimately associated with that of medicine. Indeed, it is only through the investigations into the life history of these minute forms that our present knowledge of the etiology, course and prevention of many of the infectious diseases has been acquired. The prominent position which the study of microörganisms already holds toward medicine is, moreover, continuing to increase in importance. Original discoveries are still adding to our knowledge of germ diseases, and the outlook is favorable for obtaining eventually, through serums, through attenuated cultures, or through the toxic substances produced by microörganisms themselves, means for immunizing against, if not of curing, an increased number of the specific infections. Even at present, we are able to use successfully bacterial products and protective serums as preventive or curative agents in several of the most prevalent infectious diseases. Furthermore, our knowledge concerning some microörganisms has enabled us largely to limit their dissemination and so to prevent disease. An acquaintance, therefore, with the main facts concerning pathogenic microörganisms is most necessary to the education of the modern physician.

The vast majority of the known microörganisms which cause disease belong to the closely related groups of lowest plants and animals, *i. e.*, the bacteria, the moulds, the yeasts and the protozoa. A few of the pathogenic metazoa (some of the parasitic worms) in some of their stages are also microscopic; therefore microscopic methods of study are applicable as well to them, but since they are fully presented in works on clinical microscopy they are not given here.

Before entering into a detailed consideration of the subject it may be

[1] The correct form of this word is under discussion. According to derivation—*microbe* + *logia*—the better spelling would be *microbology*, but the form given above is the one accepted at present in the dictionaries.

interesting and instructive to review very briefly a few of the important steps which led to the development of the science of microbiology and upon which its foundation rests, in which we shall see that the results obtained were gained only through long and laborious research and after many obstacles were met and overcome by accurate observation and experiment.

Possibly the first observations of living microscopic organisms of which there is any record are those of Kircher (1601–1680) in 1659. This original investigator with a very low power lens demonstrated the presence in putrid meat, milk, vinegar, cheese, etc., of "minute living worms," but did not describe further their form or character.

Not long after this, n 1675, Leeuwenhoeck[1] (1632–1723), of Delft, observed in rainwater, putrid infusions, and in his own and other saliva and diarrheal evacuations living, motile "animalcula" of most minute dimensions, which he described and illustrated by drawings. Leeuwenhoeck practised the art of lens grinding, in which he eventually became so proficient that he perfected a lens superior to any magnifying glass obtainable at that day, and with which he was enabled to see objects very much smaller than had ever been seen before. "With the greatest astonishment," he writes, "I observed distributed everywhere through the material which I was examining animalcules of the most microscopic size, which moved themselves about very energetically." The work of this observer is conspicuous for its purely objective character and absence of speculation; and his descriptions and illustrations are done with remarkable clearness and accuracy, considering the imperfect optical instruments at his command. It was not until many years later, however, that any attempt was made to define the characters of these minute organisms and to classify them systematically.

At that time all of the microscopic organisms seen were classed together as little animals. Indeed, all of the microörganisms first described at any length were probably protozoa, and only after further improvement of the lenses and a more minute study of the organisms were bacterial forms gradually recognized as a separate class. Ehrenberg (1795–1876) was probably the first to apply the names bacterium and spirillum to rod and spiral bacteria respectively.

The first ideas of the structure of the protozoa were drawn from analogy. The early observers thought that each tiny organism possessed an internal structure made up of organs and tissues similar to those in metazoa. They could not conceive of motion without articulation, tendons and muscles; nor of food absorption without an alimentary tract, and they were so impressed with the ideas of what they thought they ought to see that they were convinced that they really saw many of the complicated structures possessed by metazoa. For example, the contractile vacuole, a characteristic pulsating vesicle of the protozoa, discovered by Joblot, in 1754, was thought by many to be lungs, other vacuoles were said to be stomachs, the mouths were often seen and the rest of the alimentary tract was supplied from the imagination; the red pigment spots of many forms were interpreted

[1] Opera Omnia seu Arcana Naturæ, Leydon.

as true eyes, etc. There were many opponents to these views, however, and the idea of the cell being the unit of structure, which was advanced by Schleiden, in 1838, helped determine the fact that protozoa were single cells with no definite organic structure. With the publications of Dujardin (1835–1841) a correct idea of the structural simplicity of the microörganisms gained ground.

From the earliest investigations into the life history and properties of germs, microörganisms have been thought to play an important part in the causation of infectious diseases. Long before the discovery of pathogenic microbes the opinion was advanced that the infectious diseases were due to a "contagium vivum" and, soon after the first microörganisms were seen, so widespread became the belief in a causal relation of these minute organisms to disease that it soon amounted to a veritable craze, and all forms and kinds of diseases were said to be produced in this way upon no other foundation than that these organisms had been found in the mouth and intestinal contents of men and animals and in water. Of course, with no further evidence than this, the belief dropped into the background and only a few continued to hold it.

Among those who were especially conspicuous at this time for their advanced views on the germ theory of infectious diseases was Marcus Antonius Plenciz, a physician of Vienna. This acute observer, who published his views in 1762, maintained that not only were all infectious diseases caused by microörganisms, but the infective material could be nothing else than a living organism. On these grounds he endeavored to explain the variations in the period of incubation of the different infectious diseases. He also insisted that there were special germs for each infectious disease by which the specific disease was produced. Plenciz believed, moreover, that these organisms were capable of multiplication in the body, and suggested the possibility of their being conveyed from place to place through the air.

These views, it is true, were largely speculative, and rested upon insufficient experiment, but they were so plausible, and the arguments put forward in their support were so logical and convincing, that they continued to gain ground, in spite of considerable opposition and ridicule, and in many instances the conclusions reached have since been proved to be correct. The mode of infection, its unlimited development among large numbers of individuals, and gradual spread over wide areas—the incubation, course of, and resulting immunity in recovery from infectious diseases—all pointed to a living organism as the probable cause.

Among other distinguished men of the day whose observations exerted a most powerful influence upon the doctrine of infection, may be mentioned Henle[1] (1809–1885). His writings, in which he described the relation of microörganisms to infectious diseases and defined the character and action of bacteria upon certain phases and symptoms of these affections, are remarkable for their clearness and precision.

Origin of Micoörganisms.—The question which most interested the early explorers of the cause of infectious diseases was: Whence came

[1] Pathological Investigations, 1840, and Text-book of Rational Pathology, 1853.

these microörganisms? Were they the result of spontaneous generation, or were they reproduced from similar preëxisting organisms? The solving of this question is intimately connected with the investigations into the origin and nature of fermentation and putrefaction.

Boyle[1] (1627–1691) was one of the first to call attention to the relation of fermentation to disease. ". . . phenomena of diseases . . . will perhaps be never properly understood without an insight into the doctrine of fermentation." But it was not until much later that actual experimental demonstrations were made which showed the relationship of microörganisms to disease.

Spallanzani, in 1769, demonstrated that if putrescible infusions of organic matter were placed in hermetically sealed flasks and then boiled, the liquids were sterilized; neither were living organisms found in the solutions, nor did the infusions decompose; they remained unchanged for an indefinite period.

An objection was raised by the believers in spontaneous generation that in excluding the oxygen of the air by hermetically sealing the flasks, the essential condition for the development of fermentation, which required free admission of this gas, was interfered with. This objection was met by Schulze, in 1836, by causing the air admitted to the boiled decomposable liquids to pass through strong sulphuric acid. Air thus robbed of its living organisms did not produce decomposition.

Schwann, in 1839, obtained similar results in another way. He admitted to his boiled liquids the air deprived of microörganisms by passing it through a tube which was heated to a temperature high enough to destroy germs. To this investigator is due also the credit of having discovered the specific cause—the yeast plant, or *Saccharomyces cerevisiæ*—of alcoholic fermentation, the process by which sugar is decomposed into alcohol and carbonic acid.

Again it was objected to these experiments that the heating of the air had perhaps brought about some chemical change which hindered the production of fermentation. Schroeder and von Dusch, in 1854, then showed that by a simple process of filtration, which has since proved of inestimable value in bacteriological work, the air can be mechanically freed from germs. By placing in the mouth of the flask containing the boiled solutions a loose plug of cotton, through which the air could freely circulate, it was found that all suspended microorganisms could be excluded, and that air passed through such a filter, whether hot or cold, did not cause fermentation of boiled infusions.

Similar results were obtained by Hoffmann, in 1860, and by Chevreul and Pasteur, in 1861, without a cotton filter, by drawing out the neck of the flask to a fine tube and turning it downward, leaving the mouth open. In this case the force of gravity prevents the suspended bacteria from ascending, as there is no current of air to carry them upward through the tube into the flask containing the boiled infusion. The question of spontaneous generation still remained unsettled, however,

[1] Quoted by Nuttall in Jour. Parasitology, 1921, **13**, 706.

inasmuch as occasionally, even under the most careful precautions, decomposition did occur in such boiled liquids.

This fact was explained by Pasteur, in 1860, by experiments showing that the temperature of boiling water was not sufficient to destroy all living organisms, and that, especially in alkaline liquids, a higher temperature was required to insure sterilization. He showed, however, that at a temperature of 110° to 112° C., for one hour, which he obtained by boiling under a pressure of one and a half atmospheres, all living organisms were invariably killed. Pasteur, at a later date (1865), corroborated the fact that the organisms which resist boiling temperature are, in fact, reproductive bodies, which are known as *spores*.

These facts have been practically confirmed on a large scale in the preservation of food by the process of sterilization. Indeed, there is scarcely any biological problem which has been so satisfactorily solved or in which such uniform results have been obtained; but all through the experiments of the earlier investigators irregularities were constantly appearing.

When and how life began no one yet is able to say. Even that spontaneous generation may be taking place now under unknown conditions is conceivable, but all such ideas are purely hypothetical and there is no evidence that under present conditions any of the known microörganisms have originated in any way except from a previous similar cell.

Stimulated by the establishment of the fact, through Pasteur's investigations, that fermentation and putrefaction are due to the action of living organisms reproduced from similar preëxisting forms, and that each form of fermentation is due to a special microörganism, the study of the causal relation of microörganisms to disease was taken up with renewed vigor. Reference has already been made to the opinions and hypotheses of the earlier observers as to the microbic origin of infectious diseases. The first positive grounds, however, for this doctrine, founded upon actual experiment, were the investigations into the cause of certain infectious diseases in insects and plants. Thus, Bassi, in 1837, demonstrated that a fatal infectious malady (*pébrine*) of the silkworm was due to a parasitic protozoön. Pasteur later devoted several years' study to an exhaustive investigation into the same subject. In like manner Tulasne and Kühne showed that certain specific affections in grains, in the potato, etc., were due to the invasion of parasites, and Schoenlein showed that favus, a disease of the scalp, is due to a mould.

Very soon after this it was demonstrated that microörganisms were probably the cause of other infectious diseases in man and the higher animals. Davaine, a famous French physician, has the honor of first having demonstrated the causal relation of a microörganism to a specific infectious disease in man and animals. The anthrax bacillus was discovered in the blood of animals dying from this disease by Pollender in 1849 and by Davaine in 1850; but it was not until 1863 that the last-named observer demonstrated by inoculation experiments that the bacillus is the cause of anthrax.

The next discoveries were those relating to infections through wounds. Rindfleisch, in 1866, Waldeyer and von Recklinghausen, in 1871, Pasteur, in 1880, Ogston, in 1881, and a host of others drew attention to the minute organisms occurring in the pyemic processes resulting from infected wounds, and occasionally following typhoid fever and other diseases. Further investigations were made by Billroth, Fehleisen and others in erysipelatous inflammations secondary to injury. They agreed that in these conditions micrococci almost always could be detected in the lymph channels of the subcutaneous tissues.

The brilliant results obtained by Lister, in 1863–1870, in the antiseptic treatment of wounds to prevent or inhibit the action of infective organisms, exerted a powerful influence on the doctrine of bacterial infection, causing it to be recognized far and wide and gradually lessening the number of its opponents. Lister's methods were suggested to him by Pasteur's investigations on putrefaction.

At this time, and until comparatively recently, it was thought that pathogenic germs remained in the air for indefinite periods. Now our knowledge of germ carriers has explained many examples of supposed "air-borne" diseases. We still have to fear infecting the immediately surrounding air by expelling germs from our throat, mouth and nose in coughing, sneezing and loud talking, but we seldom need to carry out the Lister methods of antisepsis. The less strenuous methods of asepsis are usually effectual.

In 1877, Weigert and Ehrlich recommended the use of the anilin dyes as staining agents and thus made possible a more exact microscopic examination of microörganisms in cover-glass preparations.

In the year 1880 Pasteur published his discovery of the bacillus of fowl cholera and his investigations upon the attenuation of the virus of anthrax and of fowl cholera, and upon protective inoculation with attenuated germs against these diseases. In the meantime he (1865) showed that pure cultures might be obtained by the dilution method.

Laveran, in the same year, announced the discovery of parasitic bodies, the first pathogenic protozoa described in humans, in the blood of persons sick with malarial fever, and thus stimulated investigations upon the immensely important pathogenic unicellular animal parasites.

In 1881, Koch published his fundamental researches upon pathogenic bacteria. He introduced solid culture media (agar had already been used by Hess) and the "plate method" for obtaining pure cultures, and showed how different organisms could be isolated, cultivated independently, and, by inoculation of pure cultures into susceptible animals, could be made, in many cases, to reproduce the specific disease of which they were the cause. Koch's postulates, which were first formulated by Henle, a teacher of Koch, helped to further careful researches and critical examination of the proofs before any microbe was accepted as the causal organism of a disease. These are: (1) A specific organism must always be associated with a disease, (2) when isolated in pure culture (3) and inoculated into a healthy susceptible animal it must always produce the disease and (4) should be obtained again in pure culture. These were long accepted as the only proof of the causal relationship

of an organism to the disease. Later it was learned that immunological reactions add greatly to our knowledge of the specificity of microbes in disease, and can afford proof without demonstrating all of Koch's laws. It was in the course of Koch's work that the Abbé system of substage condensing apparatus was first used in bacteriology, and many other bacteriological methods were soon introduced in different parts of the world.

In 1882 Pasteur published his first communication upon rabies. The method of treatment devised by him is still in general use. A little later came the investigations of Löffler and Roux upon the diphtheria bacillus and its toxins, and that of Kitasato upon tetanus. These researches paved the way for Behring's work on diphtheria antitoxin, which in its turn stimulated investigation upon the whole subject of immunity, with Bordet, Ehrlich and Metchnikoff as chief pioneers. The number of investigators rapidly increased as the importance of the earlier fundamental discoveries became apparent. Additions to the science of microbiology have been made from many sides, and the practical application of the facts learned from these investigations is steadily increasing. These applications have been not only for the benefit of man but also for that of the higher animals and plants. The chemist too has joined in the study of microbal products and the reactions of microbes to chemicals.

In 1893, on the advice of Biggs, the first public health laboratory for the bacteriological diagnosis of infectious diseases was started in New York City. This was founded on Park's work on the diagnosis of diphtheria. Bacteriological standards for clean milk were soon formulated in this laboratory and the relationship of microbiology to public health was thoroughly established.

The results obtained during the World War constitute a striking demonstration of how much now can be accomplished, even under adverse conditions, in preventing many diseases that were earlier great scourges. For instance, typhoid, paratyphoid, smallpox and cholera were made practically *nil* by protective vaccination; tetanus was controlled by protective inoculations of antiserum; gas gangrene was lessened by special disinfectants and antiserums; plague was avoided by rat extermination; typhus and trench fever were lessened by delousing; malaria was controlled by mechanical measures, by quininization and by drainage. Since the war, the extensive work on the practical immunization against diphtheria, started by the New York City Health Department, has had a wide adoption. The stimulating discoveries of Dicks and Dochez on the etiology of scarlet fever were reported in 1923–1924.

For a more detailed account of the history of the development of bacteriology and protozoölogy in medicine see the following references:

Calkins: Protozoölogy, 1909, Lea & Febiger, New York and Philadelphia.

Löffler, Fried: Vorlesungen über die geschichtliche Entwickelung der Lehre von den Bakterien, Leipzig, F. C. W. Vogel, 1887, with bibliography.

Valleri, Radot: Life of Pasteur.

Armand-Delille, P. F.: Role of Pasteurian Methods in Prophylaxis of Infectious Diseases, Jour. Am. Med. Assn., 1918, **71**, 1809.

CHAPTER II.

CLASSIFICATION AND GENERAL CHARACTERISTICS OF MICROÖRGANISMS.

In general, the lowest forms of life are microscopic in size and unicellular in form. Many of these microscopic organisms are helpful to man,[1] while many have in common the ability to produce disease in man and other animals and in plants. Some of these microörganisms are plants, others are animals, while some are difficult to classify. Only those that are of direct importance to man are considered in this work.

CLASSIFICATION OF MICROÖRGANISMS.

The classification of microörganisms is still in the transition stage. This is due chiefly to the difficulties encountered in studying the individual morphological characteristics of such minute bodies and in determining their limits of variation.

There is no one distinctive characteristic known which separates the lowest plants from the lowest animals. While the lowest microorganisms of all—the bacteria—are usually classed as plants, their structure is so simple and their biological characteristics are so varied that their relationship to the vegetable kingdom is not clear-cut. In their possession of more or less rigid plasmolysible bodies, in the tendency of many to grow in filaments, in their division apparently by simple fission, and in the ability of some to use simple elements as food, they resemble plants; while in the motility of many, the absence of chlorophyl, and in the necessity of many for complex food, they resemble animals.

There is a similar difficulty in definitely classifying many members of the other groups of closely related microörganisms—namely, the protozoa, the yeasts and molds, and it has been suggested that under the name *Protista* a third kingdom be formed consisting of all of these lowest microörganisms.

As a rule, in the more minute organisms, genera have been based upon morphological characteristics, and species largely upon biochemical, physiological and pathogenic properties. But, owing to the fact that the morphology may vary under different conditions and that it may give no indication whatever of the relation of microörganisms to disease and fermentation—the chief characteristics of importance to human

[1] See Chapters on Intestinal Bacteria and Bacteria in the Industries, also p. 62.

beings—biochemical and related properties are being used more generally in systems of classification.

Especially in recent years has the power to produce specific antibodies been studied in relation to classification, but the constancy of these reactions and whether they may be used to indicate a fundamental chemical difference in the composition of microörganisms morphologically alike still remains to be decided.

Permanence of Species.—It is true, however, that under favorable conditions microörganisms retain most of their characteristics for an undetermined time. In the test-tube diphtheria bacilli, plague bacilli, tubercle bacilli and many others may remain the same, so far as can be determined, as when isolated thirty or more years ago, but these, under other conditions, and many forms under any observed condition, lose their virulence and other characteristics more or less quickly and sometimes apparently permanently. Thus, the power to produce spores or flagella may be held in abeyance for a time or may be lost totally; the relations to oxygen may be gradually altered, so that an anaërobic bacterium may grow in the presence of oxygen or the contrary may be the case; parasitic organisms may be so cultivated as to become saprophytic varieties, and those which, when taken from their natural habitat, have only faint traces of the power to grow in the living body may be made to acquire pathogenic properties when they are made to develop in a series of animals of the same species, whose resistance has been overcome by reducing their vitality through poisons or other means.

The work of Rosenow,[1] of Irons,[2] of Gay[3] and of others on elective localization of streptococci shows how marked may be the adaptability of certain bacteria to different conditions of growth. That the diseases are evidently the same as history shows them to have been long before the time of Christ, is not proof that the germs themselves remain the same. It is still accepted by the majority, however, that most germs which have once become established as parasites continue to produce new generations which retain their peculiar (specific) characteristics. It has been impossible to prove, however, that those which completely lose their power to invade a host and produce injury, may be made again pathogenic.

Mutations.—Morphological or biological changes, called mutations by some, have been from time to time reported, by many workers. Practically all species of bacteria minutely studied show under certain conditions the phenomena of changes in colony type and in other characteristics, some of them apparently permanent. The transition from one type to another type during disease has been noted. Such reports as those of Griffith,[4] that a pneumococcus of one type may readily be changed to another type, needs further corroboration. The recent studies on this interesting and important subject of bacterial dissociation have

[1] Jour. Am. Med. Assn., 1915, **55**, 1687.
[2] Jour. Inf. Dis., 1916, **18**, 315.
[3] Arch. Path. and Clin. Med., 1926, **1**, 510, 857.
[4] Jour. Hyg., January, 1928, vol. **27**, No. 2.

been summarized by Hadley.[1] Whether or not the changed characteristics may be in part only evidence of species characteristics cannot at present be decided. How much bacterial autolysins may have to do with lasting variations is under investigation.[2] (See below.)

WORKING CLASSIFICATION OF GENERA CONTAINING CHIEF MICRO-ORGANISMS PARASITIC IN MAN (AND INCLUDING OTHER FORMS IMPORTANT IN DIAGNOSIS).

Kingdom.	Philum.	Class.	Order.	Family.	Genus.
Plants	Thalophyta	Schizomycetes (Bacteria)	Eubacteriales	Coccaceæ	Staphylococcus, Streptococcus, Diplococcus, Neisseria.
				Bacteriaceæ	Pseudomonas, Lactobacillus, Escherichia, Aërobacter, Proteus, Salmonella, Eberthella, Alkaligines (Brucella), Encapsulatus Pasteurella, Klebsiella, Hemophilus.
				Bacillaceæ	Bacillus, Clostridium.
				Spirillaceæ	Vibrio.
			Actinomycetales	Mycobacteriaceæ	Corynebacterium, Fusiformis, Pfeifferella, Mycobacterium.
				Actinomycetaceæ	Actinobacillus, Leptotrichia, Actinomyces, Erysipelothrix.
			Spirochetales	Spirochetaceæ	Borrelia, Treponema, Leptospira.
		Hyphomycetes (Fungi imperfecti; asci absent; mycelium septate)	Thalosporales	Cryptococcaceæ	Cryptococcus, Ptyrosporum, Torula.
				Oösporaceæ	Monilia, Oïdium.
			Chidosporales	Sporotrichinaceæ	Sporotricum.
				Trichophytonaceæ	Trichophyton, Microsporum, Achorion, Endodermophyton, Epidermophyton.
		Ascomycetes (Asci present; mycelium septate)	Saccharomycetales	Saccharomycetaceæ	Saccharomyces, Willia, Deparyomyces.
				Endomycetaceæ	Coccidioides.
			Aspergillales	Aspergillaceæ	Aspergillus, Penicillium.

[1] Newer Knowledge, Univ. of Chicago Press, 1928.
[2] Evans: Jour. Bact., 1929, **17**, 63.

Kingdom. Philum.	Class.	Order.	Family.	Genus.
Plants Thalophyta	Phycomycetes (Asci absent; mycelium non-septate in vegetative stage)	Mucorales	Mucoraceæ	Mucor.
Animals Protozoa	Mastigophora (Flagellates)	Protomonadina	Trypanosomidæ (Binucleata)	Herpetomonas, Leishmania, Crithidia, Trypanosoma (Castellanella, Duttonella), Schizotripanum.
			Tetramitidæ Octomitidæ	Chilomastix, Trichomonas Giardia.
		Rhizomastigina		Mastigamœba.
	Sarcodina (Amœbas)	Lobosa	Amœbidæ	Endamœba, Endolimax, Iodamœba, Dientamœba.
	Sporozoa	Coccidia		Eimeria, Isospora.
		Hemosporidia	Plasmodidæ Piroplasmidæ Hemogregarinidæ	Plasmodium (Hemamœba). Piroplasma (Babesia). Hemogregarina.
		Sarcosporidia		Sarcocystis.
	Infusoria (Ciliates)	Heterotrichia		Balantidium, Nyctotherus.

The report published by the Society of American Bacteriologists[1] on the classification of bacterial types, and Bergey's[2] compilation also are helps in showing us how little we still know. It therefore seems wiser for us to give again only a broad simple grouping and refer the student to the bibliography for further information in regard to attempts at classification, and the laws to be observed in identifying and naming a new organism. (See also Large Table, also Descriptive Chart.)

[1] The Families and Genera of the Bacteria, Jour. Bact., 1920, 5, 191.
[2] Determinative Bacteriology, Baltimore, 1925.

While we are recognizing the theory that bacteria are related, on the one hand, to the higher plants through the actinomycetes and, on the other hand, to the animal kingdom through the spirochetes, for the sake of convenience in teaching, the organisms will not be taken up in the book in just this order.

GENERAL CHARACTERISTICS OF EACH GROUP OF MICROÖRGANISMS.

Introduction.—The knowledge gained from the fact that each variety of microbe possible of cultivation may grow in distinctive ways upon so-called artificial culture media has been an immense aid in studying the characteristics of the more minute forms, for the individual cell of most varieties among the bacteria is so minute that even the highest magnification we have may show little if any morphological difference between organisms which produce distinctly different diseases, or between a pathogenic and a non-pathogenic form. There are, however, certain group morphological and biological characteristics of the individual cell, which are pronounced; we therefore study these before going on to the study of cultures, that is, of microörganisms in masses.

BACTERIA.

Definition.—*Bacteria* may be defined as extremely minute, simple, unicellular microörganisms, which reproduce themselves under suitable conditions with exceeding rapidity, usually by transverse division, and grow without the aid of chlorophyl. They have no morphological nucleus, but contain nuclear material which is generally diffused throughout the cell body in the form of larger or smaller granules.

Natural Habitat.—Bacteria are found all over the known world. Wherever there is sufficient moisture, one form or another will find conditions adequate for multiplication. Thus, we meet with bacterial life between 0° and 75° C. Some bacteria live only in the tissues of man, others in lower animals, a small number may grow in both man and lower animals, still others grow only in plants, but by far the greatest number live in dead organic matter. For some, free oxygen is necessary to life; for others, it is a poison.

Morphology (see Plates I and II).—The form and dimensions of bacterial cells at their stage of complete development must be distinguished from those which they possess just after or just before they have divided. As a spherical cell develops preparatory to its division into two cells it becomes elongated and appears as a short oval rod; at the moment of its division, on the contrary, the transverse diameter of each of its two halves is greater than their long diameter. A short rod becomes in the same way, at the moment of its division, two cells,

the long diameter of each of which may be at first equal to or even a trifle less than the short diameter, and thus they may appear on superficial examination as spheres.

Size.—The dimensions of the adult individual vary greatly in the different species as well as in members of the same species. The largest bacillus recorded is 50μ[1] to 60μ long and 4μ to 5μ wide (B. bütschlii, see Fig. 8, p. 38). One of the smallest forms known (B. influenzæ) has an average size of 0.5μ x 0.2μ. A rough idea may be gained of the size of bacteria by comparing it with that of a human red blood cell.

Some pathogenic organisms are so small (ultramicroscopic, see p. 73, also chapter on Filtrable Viruses) as to be invisible with any magnification which we now possess. We know of their existence either by the fact that they may be cultivated in artificial media, producing appearances of mass growth, and that such cultures when inoculated into susceptible animals cause the characteristic disease, or by the fact that the filtrates, after passing through the pores of the finest filters, are infectious. A number of minute organisms just visible at a magnification of 1500 diameters, not well studied, may pass the coarser porcelain filters.

Shape.—The *basic forms* of the single bacterial cells are threefold—the sphere, the rod and the segment of a spiral. Although under different conditions the type form of any one species may vary considerably, yet these three main divisions under similar conditions are constant, and, so far as we know, it is never possible to bring about changes in the organisms that will result in the *permanent* conversion of the morphology of the members of one group into that of another—that is, micrococci always, under suitable conditions, produce micrococci, bacilli produce bacilli, and spirilla produce spirilla. (See p. 25.)

As bacteria multiply, the cells produced from the parent-cell have a greater or less tendency to remain attached. This is due to the tenacious covering which is more or less developed in all bacteria. This union may appear simply as an aggregation of separate bacteria or so close that the group appears as a single cell. According to the method of the cell division and the tenacity with which the cells hold together, there are different groupings of bacteria, which aid us in their differentiation and identification. Thus, in cocci we get the bacterial cell dividing into one, two, or three planes (Plate I, Figs. 1 to 5), while in bacilli and spirilla the division is generally in only one plane (Plate I, Figs. 6 to 9).

1. Spherical Form, or Coccus.—The size varies from about 0.15μ as minimum diameter to 3μ as maximum; the average size of the pathogenic cocci is 0.8μ. The single elements are, at the moment of their complete development, so far as we can determine, practically spherical; but when seen in the process of multiplication through division the form is seldom that of a true sphere. Here we have elongated or lancet-shaped forms, as frequently seen in the diplococcus of pneumonia, or the opposite, as in the diplococcus of gonorrhea, where the cocci appear

[1] A μ, or micromillimeter, is $\frac{1}{25000}$ of an inch.

to be flattened against one another. Those cells which divide in one direction only and remain attached are found in pairs (diplococci) or in shorter or longer chains (streptococci). Those which divide in two directions, one at right angles to the other, form groups of four (tetrads). Those which divide in three directions and cling together form packets in cubes (sarcinæ). Those which divide in any axis form irregularly shaped, grape-like bunches (staphylococci).

2. Rod Form, or Bacillus.—The type of this group is the cylinder. The length of the fully developed cell is always greater than its breadth. The size of the cells of different varieties varies enormously—from a length of 30μ and a breadth of 4μ to a length of 0.2μ and a breadth of 0.1μ. The largest bacilli met with in disease do not, however, usually develop over 3μ x 1μ, while the average is 2μ x 0.5μ. Bacilli are roughly classed, according to their form as slender when the ratio of the long to the transverse diameter is from 1 to 4 to 1 to 10, and as thick when the proportion of the long to the short diameter is approximately 1 to 2.

The characteristic form of the bacillus has a straight axis, with uniform thickness throughout, and flat ends; but there are many exceptions to this typical form. Thus, frequently certain bacilli have rounded ends, many of the more slender forms have the long axis slightly bent; some few species, as for example the diphtheria bacilli, invariably produce many cells whose thickness is very unequal at different portions. Spore formation also causes an irregularity of the cell outline.

The bacilli, except when they develop from spores or granules, divide only in the plane perpendicular to their long axis. A classification of bacilli, therefore, according to their manner of grouping is much simpler than in the case of the cocci. We may thus have bacilli as isolated cells, as pairs (diplobacilli), or as longer or shorter chains (streptobacilli).

EXPLANATION OF PLATE I.

Fig. 1.—Illustrating cocci single or in irregular groups (micrococcus, staphylococcus), micrococcus from air. × 1000.

Fig. 2.—Illustrating cocci in twos—diplococcus pneumoniæ from peritoneal exudate of rabbit. × 1000.

Fig. 3.—Illustrating cocci in chains—Streptococcus pyogenes. × 1000.

Fig. 4.—Illustrating cocci in fours—Micrococcus tetragenus from spleen of mouse, × 1000.

Fig. 5.—Illustrating cocci in packets—Sarcina lutea from air. × 1000.

Fig. 6.—Illustrating large single bacilli: Bacillus subtilis (hay bacillus). × 1000.

Fig. 7.—Illustrating small bacilli; mostly in twos—Bacillus hoffmanni from human throat. × 800. (Park.)

Fig. 8.—Illustrating bacilli in chains—anthrax bacillus from spleen of mouse. × 500.

Fig. 9.—Illustrating bacilli in bunches—typhoid bacillus from human spleen. × 500.

Fig. 10.—Illustrating bacilli in threads—anthrax bacilli from blood of frog.

Fig. 11.—Spirillum undula, showing flagella. × 1000.

Fig. 12.—Cholera spirilla, gelatin culture. × 1000.

Fig. 13.—Large spirilla in chains from water. × 1000.

Fig. 14.—Smaller spirilla in chains—Spirillum rubrum. × 1000.

Fig. 15.—Streptothrix candida—broth culture (Zettnow, from Kolle and Wassermann).

Fig. 16.—Streptothrix hominis from sputum (Zettnow, from Kolle and Wassermann).

Unless otherwise indicated, the photographs are from Fränkel and Pfeiffer.

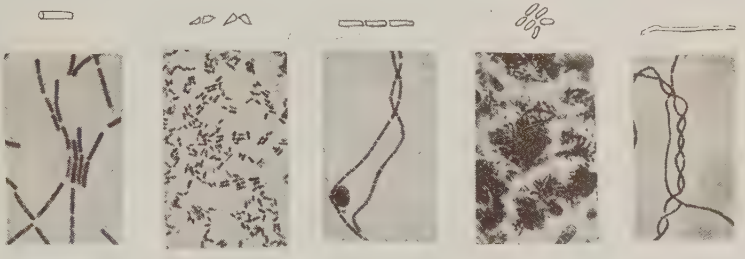
PLATE I

TYPES OF BACTERIA

I SPHERE (COCCI OR COCCACEÆ)

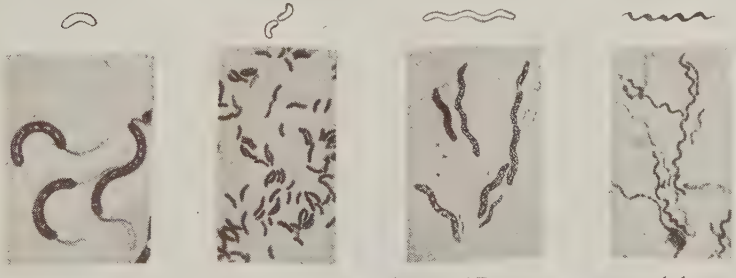
1 2 3 4 5

II CYLINDER (BACILLI OR BACTERIACEÆ)



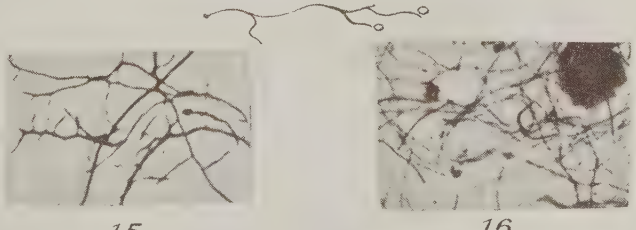
6 7 8 9 10

III SPIRAL (SPIRILLA OR SPIRILLACEÆ)



11 12 13 14

IV HIGHER BACTERIA (TRICHOBACTERIA)



15 16

3. Spiral Form, or Vibrio.—The members of the third morphological group are spiral in shape, or only segments of a spiral. Here, too, we have large and small, slender and thick spirals. The twisting of the long axis, which here lies in two planes, is the chief characteristic of this group of bacteria. Under normal conditions the twisting is uniform throughout the entire length of the cell. The vibrio, like the bacilli, divide only in one direction. A single cell, a pair, or the union of two or more elements may thus present the appearance of a short segment of a spiral or a comma-shaped form, an S-shaped form, or a complete spiral or corkscrew-like form.

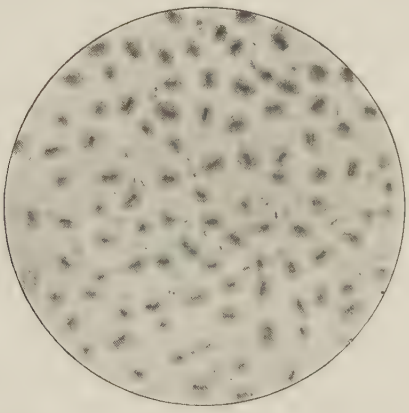


Fig. 1.—Bacillus pneumoniæ (Bacillus mucosus) stained for capsule by Huntoon's method. × about 900. (Huntoon.)

Structure of Bacterial Cells.—When examined living in a hanging drop (see p. 74) under the microscope, bacteria appear usually as colorless refractive bodies with or without spores or other more highly refractive areas. It is only by the use of stains that we are able to see more of their structure. Spores are described under Physiological Characteristics, p. 38.

Capsule.—Special staining methods (see p. 84) show that many bacteria (some investigators say all) under certain conditions possess a *capsule* (Plate II, Figs. 14 to 16), a gelatinous envelope which is supposed to be formed from the outer layer of the cell membrane. Some bacteria easily develop a much thicker capsule than others. Such forms are known as capsule bacteria. These generally produce a slimy growth on cultivation, *e. g.*, B. (Klebsiella) mucosus.

Capsules develop best in animal tissues. In cultures, with a few exceptions, they require for their development special albuminous culture media, such as milk, blood serum, bronchial mucus, etc. In ordinary nutrient media or on potatoes the capsule may be visible in the first culture generations when grown from the body, but usually it shows very indistinctly, if at all. The capsule is distinguished by a diminished power of staining with ordinary aniline dyes; therefore,

unless special staining methods are used, the bacteria may appear to be lying in a clear unstained area. With certain dyes the inner portion of the capsule stains, giving the bacteria an apparent greater diameter. The demonstration of the capsule is often of help in differentiating between closely related bacteria; *e. g.*, some forms of streptococcus and pneumococcus. The relationship between virulence and capsule formation is not yet decided.

Cell Membrane.—That all bacteria possess a cell membrane is shown (1) by special staining methods (*e. g.*, flagella stains, see p. 87) and (2) by plasmolysis, demonstrated by placing the bacteria in a 1 per cent. solution of sodium chloride when the central portion (entoplasm?) contracts and separates in places from the membrane (see also p. 61) under influence of pressure. In some bacteria the membrane is less developed than in others. It is different in composition from the membrane of higher plants in not possessing cellulose; in some forms, however, a similar carbohydrate, hemicellulose, has been demonstrated. In certain forms a substance related to chitin, found in the cyst walls of protozoa, has been found. Some observers consider the cell membrane merely a concentrated part of the cytoplasm, similar to the ectoplasm of higher cells. That it is closely related to the living part of the cell is shown by the connection of the organs of locomotion (flagella) with it (Plate II, Figs. 17 and 21).

EXPLANATION OF PLATE II.

Partly schematic (Williams). Stained by Giemsa except where otherwise indicated.

FIGS. 1 to 4.—Anthrax bacilli at different stages of development.

FIG. 1.—Shows chromatin-staining masses (red), in plastin-staining cells (light blue).

FIG. 2.—Shows same organism after one hour on fresh medium, chromatic substance distributed throughout medium and whole organism taking deeper, more homogeneous stain.

FIG. 3.—Organism showing two kinds of granules according to staining powers.

FIG. 4.—Same organism placed on fresh medium extrudes some granules (waste products) and redissolves others (chromatin granules).

FIGS. 5 to 9.—Similar granules in diphtheria bacilli at various stages of development.

FIG. 10.—Metachromatic granules in Bacillus pyocyaneus, Neisser stain.

FIG. 11.—Metachromatic granules in sarcinæ, Neisser stain.

FIG. 12.—Metachromatic granules in Bacillus influenza, Giemsa's stain.

FIG. 13.—Metachromatic granules in gonococci, Giemsa's stain.

FIG. 14.—Pneumococcus, capsule stained by Hiss method.

FIG. 15.—Pneumococcus mucosus (Streptococcus mucosus) by Welch stain for capsule.

FIG. 16.—Rhinoscleroma bacillus by Hiss stain for capsules.

FIG. 17.—Plasmolysis in cholera bacillus showing capsule.

FIGS 18 to 21 inclusive.—Types of flagella by Löffler stain.

FIG. 18.—Monotricha, cholera vibrio.

FIG. 19.—Amphitricha, water bacillus.

FIG. 20.—Lophotricha, spirillum undula.

FIG. 21.—Peritricha, typhoid bacillus.

FIG. 22.—Formation and end germination of spores in anthrax.

FIG. 23.—Lateral germination of spore in Bacillus subtilis.

FIG. 24.—Central germination of spores in Bacillus alvei (Wilson).

FIG. 25.—Type of spores: *A*, central; *B*, eccentric; *C*, end.

FIG. 26.—Diphtheria bacilli in old cultures, Giemsa's stain.

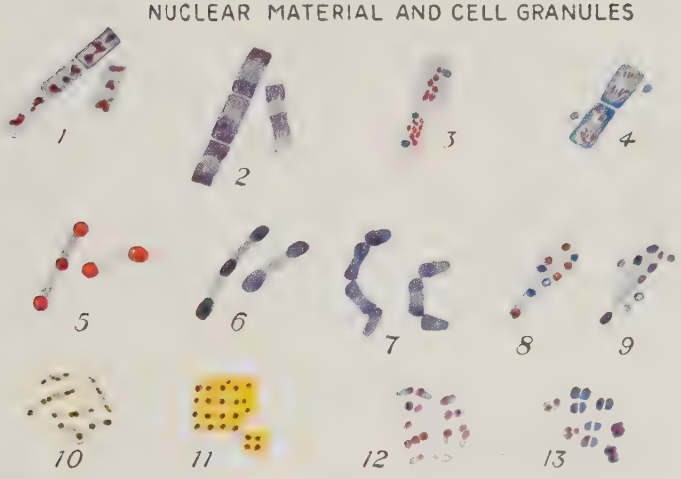
FIG. 27.—Plague bacilli in old cultures, stained by methylene blue.

FIG. 28.—Influenza bacilli in old cultures, Giemsa's stain.

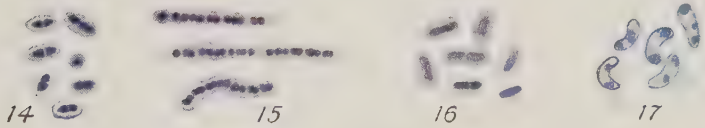
PLATE II

STRUCTURE OF BACTERIA

NUCLEAR MATERIAL AND CELL GRANULES

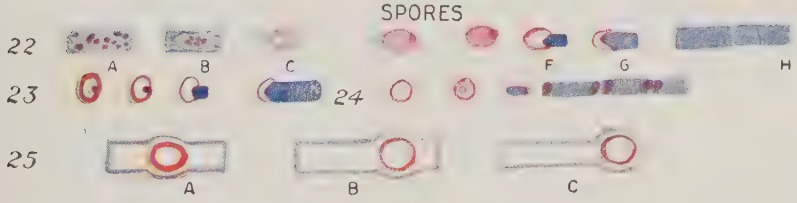


CAPSULES AND MEMBRANES

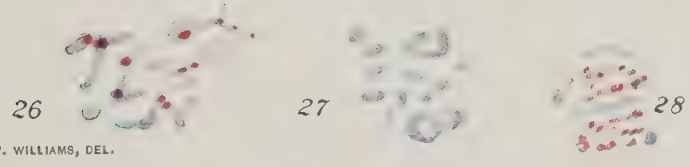


FLAGELLA

SPORES



IRREGULAR FORMS IN OLD CULTURES (INVOLUTION FORMS)



A. W. WILLIAMS, DEL.

The Cell Substance.—The chief views in regard to the nature and the structure of the cell substance contained within the membrane may be summarized as follows:

1. Bacteria have a definite morphological, more or less centrally situated nucleus (Feinberg, Nakanischi, Shottelius, Swellengrebel and others).
2. Bacteria have no nucleus or differentiated nuclear material (Fischer, Migula, Massart and others).
3. The whole organism, except the membrane which is a delicate layer of cytoplasm, is a nucleus (Bütschli, Löwit, Boni and others).
4. The nuclear material is in the form of chromatin granules distributed throughout the cytoplasm (Hertwig, Schaudinn, Guilliermond, Zettnow and others).
5. A variety of the fourth view is that bacteria possess both chief elements of a cell, namely, cytoplasm and karyoplasm, but that these are so finely mixed that they cannot be morphologically differentiated (Weigert, Mitrophanow, Gotschlich).
6. Another view advanced, which is a variation of views 3, 4 and 5, is that the bacterial cell is a relatively simple body—a cytode in Haeckel's sense, or the plasson of Van Beneden—which possesses both chromatin and plastin, the relative amounts of these chief substances of a cell corresponding more to the amounts found in the nuclei of higher cells than in their cytoplasm (Ruzicka,[1] Ambrôz[2]).

The last two authors call attention to the fact that both nucleus and cytoplasm in the higher cells are composed of a mixture of chromatin and plastin and that the chief difference between the two mixtures is one of amount and not of kind.

Our own studies of the structure of bacteria lead us to agree with the views expressed in Nos. 4 and 6 of the above summary—that is, bacteria possess both chief elements of a cell, namely, chromatin and plastin, and according to the stage of growth and division (varying with species) the chromatin may be in the form of morphological granules or may be so finely divided and mixed with the plastin as to be indistinguishable from it. (See Plate II, Figs. 1, 2, 5, 6, 7.)

Metachromatic Granules (Plate II, Figs. 1 to 13).—These granules appear in unstained bacteria as light-refracting, in stained preparations, as deeply stained areas. They have a great affinity for dyes, and so stain readily and give up the stain with some difficulty. With complex stains they show a greater affinity than the rest of the bacillus for certain constituents of the stain—*e. g.*, with polychromic methylene blue they take up more of the azure, thus appearing red as does the stained nuclein in morphological nuclei. In certain bacteria, such as the diphtheria bacilli, they are especially well marked in young, vigorous cultures. Here they have diagnostic value. At least some of these granules are nuclear in character.

Other Bacterial Granules.—Certain other granules which take up stains readily, and others still which absorb stains with difficulty, are of the

[1] Cytologie der sporenbildenden Bakterien, etc., Centralbl. f. Bakt., 1909, II. Abt., vol. **27**.

[2] Entwickelungszyklus des B. nitri n. sp., etc., Centralbl. f. Bakt., etc., I. Abt., orig., 1909, **51**, 193 (with bibliography on structure and development of bacteria).

nature of starch, of fat or of other food products. Meyer[1] has described some as being composed of volutin, a protein characterized by insolubility in alcohol and solubility in water, acids and alkalis. Certain saprophytic forms have sulphur, others iron granules.

Organs of Motility.—The outer surface of spherical bacteria is almost always smooth and devoid of appendages; but that of the rods and spirals is frequently provided with fine, hair-like appendages, or *flagella*, which are organs of motility (Plate II, Figs. 18 to 21). These flagella, either singly or in tufts, are sometimes distributed over the entire body of the cell, or they may appear only at one or both ends of the rod. The polar flagella appear on the bacteria shortly before division. The flagella, according to certain investigators, are formed from the outer cell layer (ectoplasm) or from the membrane or capsule, according to others, they arise in endoplasmic granules. So far as we know, the flagella are the only means of locomotion possessed by the bacteria. They are not readily stained, so special staining agents are required for

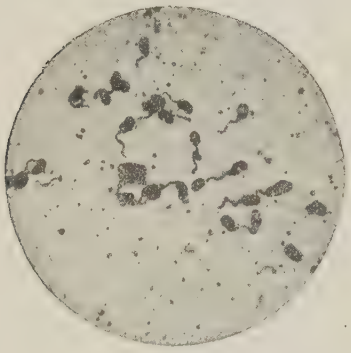


FIG. 2.—Bacilli showing one polar flagellum.

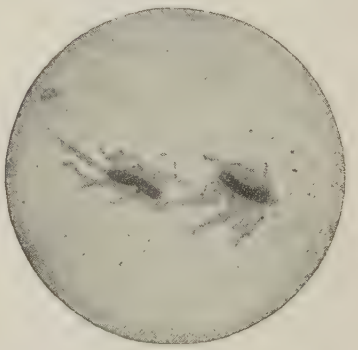


FIG. 3.—Bacilli showing multiple flagella.

this purpose (see p. 87). The envelope of the bacteria, which usually remains unstained with the ordinary dyes, then becomes colored and more distinctly visible than is commonly the case. Occasionally, however, some portion of the envelope remains unstained, when the flagella present the appearance of being detached from the body of the bacteria by a narrow zone. Flagella are best demonstrated in young cultures grown in a fluid medium at optimum temperature. In stained cultures of richly flagellated bacteria peculiar plaited masses sometimes are observed consisting of flagella which have been detached and then matted together. Bacteria may lose their power of producing flagella for a series of generations. Whether this power is then permanently lost or not we do not know.

Bacteria are named according to the number and position of the flagella[2] they possess as follows: *Monotricha* (a single flagellum at one

[1] Flora, 1908, p. 95.

[2] Messea (1891) called those bacteria possessing no flagella, Gymnobacteria, and those possessing flagella, Trichobacteria.

pole, *e. g.*, cholera spirillum); *amphitricha* (a flagellum at each pole, *e. g.*, many spirilla); lophotricha (a tuft of flagella at one pole,[1] *e. g.*, Spirillum undula); *peritricha* (flagella projecting from all parts of surface, *e. g.*, B. typhosus, and others) (Plate II, Figs. 18–21).

So far, in only a few bacteria (*e. g.*, the largest spirilla) have flagella been demonstrated during life, and then only under special conditions (see K. Reichert for bibliography). We have, however, an organism belonging to the B. alvei group, which shows its flagella very distinctly during life when a small portion of the viscid growth in a liquefying Löffler blood-serum tube is transferred to a hanging mass of agar (p. 75) and examined under high magnification. The flagella on this organism may be seen also with dark-field illumination. Reichert claims that all motile bacteria show their flagella by this method.

Physiological Characteristics of Bacteria.—The essential physiological activities of bacteria are: motility, growth, reproduction, and spore formation.

Motility.—Many bacteria, when examined under the microscope, are seen to exhibit active movements in fluids. The movements are of a varying character, being described as rotary, undulatory, sinuous, etc. Definite progressive movement may be very rapid, or so slow that it may be difficult to say positively whether there is any actual motility or whether the organism shows only molecular movements—so-called "*Brownian*" movements, or *pedesis*—a dancing, trembling motion possessed by all finely divided organic particles. In order to decide definitely with regard to the motility of any bacterial preparation, it is well to make two hanging drops. To one, 5 per cent. of formalin is added, which, of course, kills the organism. If, now, the live culture shows motility, which is not shown by the killed culture, it is an indication that one is dealing with a motile culture. Young cultures transplanted from young cultures in a suitable fluid medium and grown at the optimum temperature are most actively motile. If the surroundings are unfavorable the organism may exhibit no motility; hence one should examine cultures under various conditions before deciding as to the non-motility of any organism.

The highest speed of which an organism is capable has been approximately estimated with some forms, and the figures show an actual slow rate of movement, though, comparatively, when the size of the organism is considered, the movement is rapid. Thus, the cholera spirillum may travel for a short time at the rate of 18 cm. per hour.

Taxis.—Movement is influenced by many factors, such as chemicals (the oxygen in the air especially), heat, light and electricity. The tactile property which enables microörganisms to take cognizance of various forces is known as *taxis*; when forces attract, the phenomenon is known as positive taxis and when they repel, it is called negative taxis. Chemotaxis, or the effect of chemicals, is taken up in detail on page 59.

[1] Some investigators consider that every flagellum is essentially a tuft, composed of many small fibrils.

Growth and Reproduction.—Under favorable conditions bacteria grow rapidly to a certain size, more or less constant for each species, and then divide by fission into approximately equal halves. The average time required for this process is twenty to thirty minutes. Probably in all species the nuclear material divides first. This is certainly the case in the group to which the B. diphtheriæ belongs, where division of the nuclear granules may be observed in the living organism before the characteristic snapping of the cell body and where division into equal halves seldom occurs.

Fig. 4.—Successive stages in division of Bacillus diphtheriæ, showing relation of line of division to metachromatic granule. Continuous observation of living bacillus drawn without camera lucida. (Williams.)

According to our observations on the living cell of members of this group, division takes place at a point occupied by a metachromatic granule (Fig. 4). Before division of the cell body the metachromatic granule, which appears to contain nuclear substance, elongates and shows a darker line at or near its center. This seems to divide and form two lines, each of which has at a point near the surface a very tiny, refractive granule, staining deeply with chromatin stains. Between these two lines the cell body suddenly divides with a snap, like the opening of a jack-knife, division beginning at the point between the two tiny granules, and the two new cells remain for a variable time attached at opposite points, thus giving the V-shaped forms. Kurth and Hill also called attention to division by snapping in members of the diphtheria-bacillus group, though neither recognized the relation between the position of the metachromatic granules and the point of division. The tiny granules are probably similar to the cell-partition granules described by various observers, as occurring in metazoan cells.

Growth Curve.—It is not often that the favorable conditions mentioned above for the production of equal and rapid division obtain for any length of time, since even in pure cultures bacteria in their growth soon produce an environment unfavorable for further multiplication. Among the factors which make this environment are: (1) The using up of suitable food and moisture; (2) the disintegration of food substances into various injurious products, such as acids, alkalis and lytic substances; (3) in mixed cultures the overgrowth of one or more varieties. As these unfavorable conditions are more or less constantly present, we seldom see such absolute symmetry in the growth and division of bacteria as is usually described. In fact, even under ideally favorable conditions (*e. g.*, rapid successive transfers from young cultures on the most favorable food medium), we would never see absolutely equal fission among bacteria, and in some species, notably the diphtheria group, division is extremely irregular even in our usual twenty-four hour cultures on favorable media at favorable temperature.

Involution and Degeneration Forms.—It follows, from the conditions considered above, that, as cultures grow older or when media unfavorable to equal division are used, the bacteria may show extremely irregular forms, absolutely different from the young forms, such as long

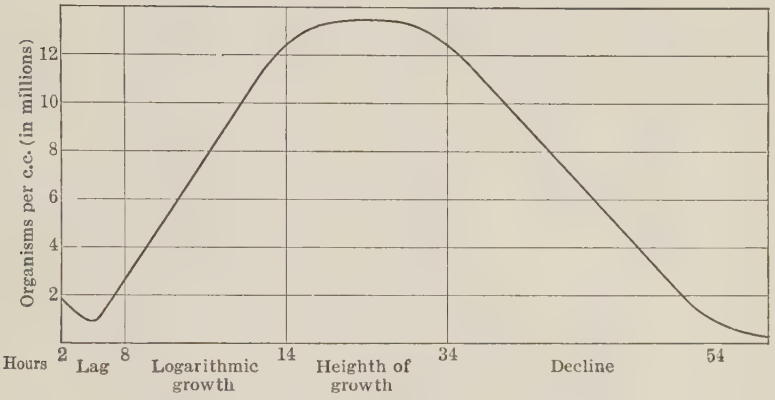


FIG. 5.—Typical rate of growth curve of certain bacteria.

threads or filaments with irregular thickenings, coccus forms from bacilli and spirilla which have divided without increasing in length, bacillar forms from cocci which have grown without dividing, and apparently branched forms from many varieties of bacilli and spirilla. These have been called *involution* or *degeneration forms.*

FIG. 6.—Bacillus diphtheriæ "No. 8" from nine days' broth pellicle, showing many "branched" forms. Stained with carbolfuchsin. × 1500 diameters.

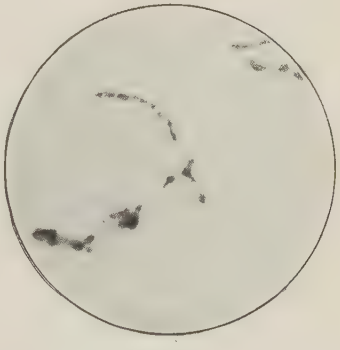


FIG. 7.—Bacillus diphtheriæ "No. 8" from ten days' broth pellicle, showing longitudinal fusion and position of metachromatic granules. Stained with Löffler's methylene blue. × 2000 diameters.

In our study of the so-called branched forms of the diphtheria bacillus we have observed the following interesting fact: Under certain conditions, marked apparent branching appears at a definite time in the age of the culture. The conditions are: slightly disturbed growth in pellicle on nutrient broth. When

such pellicles are examined every day they are found to contain, on the sixth to the twelfth day, varying chiefly with the amount of disturbance, many large intensely staining forms with one to several apparent branches and many large metachromatic granules (Figs. 6 and 7). The facts that these forms were the only ones to show active growth and division when examined on a hanging mass of agar and that in such growth the metachromatic granules seem to fuse (Fig. 8) before fission leads us to suppose that these forms represent a primitive sexual process, a sort of autogamy. Schaudinn[1] (Fig. 8) has reported a primitive conjugation (autogamy) and a relationship between the chromatin granules, or nuclear substance, and the spores in certain bacteria.

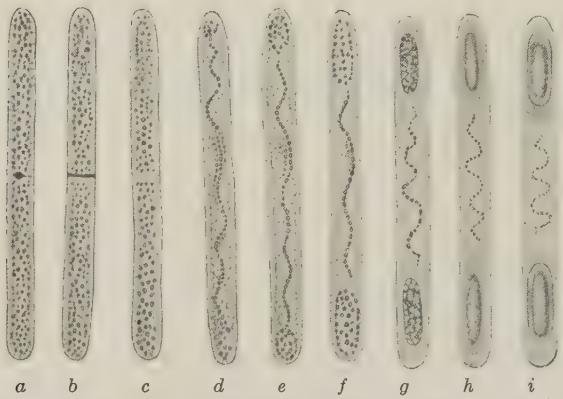


FIG. 8.—Bacillus bütschlii: *a* to *c*, incomplete division of the cell; *d* to *f*, gradual collection of chromatin granules at ends of cells; *g* to *i*, formation of end spores from these chromatin end masses. (After Schaudinn.)

Irregularities in division during the regular life cycle of the typhoid-dysentery group have been reported by Hort and others.

Incomplete Segmentation.—Although elongation in the greater diameter and complete division at right angles to this is the rule for the majority of bacteria, there are certain groups, which, instead of becoming separated from each other as single cells, tend to produce an incomplete segmentation, the cells remaining together in masses—as the sarcinæ, for example, which divide more or less regularly in three directions. The indentations upon these masses or cubes, which indicate the point of incomplete fission, give to these bundles of cells the appearance of a bale of materials. The rod-shaped bacteria have never been seen to divide longitudinally.

Spore Formation.—The formation of spores is the process by which the organisms are enabled to enter a stage in which they resist deleterious influences to a much greater degree than is possible in the growing or vegetative condition. It is true that in all non-spore-bearing cultures a certain proportion of the bacteria are more resistant than the average. No marked difference in protoplasm, however, has been noted in them other than the ability to stain more intensely and sometimes to show strong metachromatic areas. The difference between the resistance of

[1] Beiträge zur Kenntnis der Bakterien, etc., Arch. f. Protistenk, 1902, **1**, 306, and 1903, **2**, 416.

these and the less resistant forms is not great. Some have believed that this resistance is due to certain bodies called *arthrospores*, which are abnormally large cells with, usually, a thickened cell wall and increased staining properties, formed as a rule in old cultures. Foulerton and others have described similar forms in some of the higher bacteria and consider them spores.

The *true spores* (endospores) of the bacteria are definite bodies. These are strongly refractile and glistening in appearance, oval or round in shape, and composed of concentrated protoplasm developed within the cell and surrounded by a very dense envelope (Plate II, Figs. 22 to 25). They are characterized by their power of resisting the injurious influences of heat, desiccation, and chemical disinfectants up to a certain limit (see chapter on Disinfection). Spores also stain with great difficulty. (See page 85 for details.)

The production of endospores in the different species of bacteria, though not identical in every instance, is very similar. The conditions under which they are produced in nature are supposed to be similar to those observed in artificial cultures, but they may not always be similar, hence we must not consider as absolutely settled that a bacterium is a non-sporebearer because in the laboratory it has not been seen to form spores. Usually in laboratory cultures the formation of spores is best observed in an aërobic streak culture on nutrient agar or potato, which should be kept at the temperature nearest the optimum for the growth of the organism to be examined. At the end of twelve, eighteen, twenty-four, thirty, thirty-six hours, etc., specimens of the culture are observed, first unstained in a hanging drop or on an agar mass, and then, if round or oval highly refractile bodies are seen, stained for spores. Each bacillus, as a rule, produces but one spore. Only a few have reported seeing two spores and their observations were made early in the history of bacteriology.[1]

Motile bacteria usually come to a state of rest or immobility previous to spore formation. Several species first become elongated. The anthrax bacillus does this, and a description of the method of its production of spores may serve as an illustration of the process in general (Plate II, Fig. 22, *A–E*). In the beginning, the protoplasm of the elongated filaments is homogeneous, but after a time it becomes turbid and finely granular. These fine granules are then replaced by a smaller number of coarser granules, the so-called sporogenous granules, supposed to be chiefly nuclear in nature, which, by coalescence, finally amalgamate into a spherical or oval refractive body. This is the spore. As soon as the process is completed there may appear between each two spores a delicate partition wall. For a time the spores are retained in a linear position by the cell membranes of the bacilli, but these are later dissolved or broken up and the spores are set free. Not all the cells that make the effort to form spores, as shown by the spherical bodies contained in them, bring these to maturity; indeed, many varieties, under

[1] Koch: Bact. Ztschr., 1888; Frenzel: Ztschr. f. Hyg., 1891, **11**, 207; Mend. Centralb. f. Bakt. II, 1904, **12**, 559.

certain cultural conditions (*e. g.*, high temperature), seem to lose altogether their property of forming spores. This is notably true for the anthrax bacillus.

The following are the most important spore types: (*a*) the spore lying in the center of the cell, which may be much distended in its central portion, giving it a spindle shape or clostridium, *e. g.*, Bacillus (clostridium) butyricus; (*b*) the spore lying at the extremity of a cell much enlarged at that end—the so-called "head spore" or plectridium, *e. g.*, the tetanus bacillus; (*c*) the spore lying eccentrically, *e. g.*, B. (clostridium) œdemati (Plate II, Fig. 25, *A–C*).

According to Schaudinn[1] and others, in certain spore-bearing bacteria the spore formation is part of a sexual-like process. (See under Reproduction.)

The *germination of spores* takes place as follows (Plate II, Figs. 22–24):—By the absorption of water they become swollen and pale in color, losing their shining, refractive appearance; later, a little protuberance is seen upon one side (equatorial or central germination) or at one extremity of the spore (polar germination), and this grows out to form a rod which consists of soft-growing protoplasm enveloped in a membrane, which is formed of the endosporium or inner layer of the cellular envelope of the spore. The outer envelope, or exosporium, is either cast off, when it may be seen in the vicinity of the newly formed rod, or it may be absorbed, as is often the case after central germination.

The chief spore formers among the pathogenic bacteria are the anaërobes (bacillus of tetanus, of malignant edema, certain intestinal bacteria). Only one distinctly pathogenic aërobe is known to produce endospores—the anthrax bacillus.

The Higher Forms of Bacteria (see also Part II).—Some forms grow out into branching threads and thus make a group of organisms intermediate between bacteria and the moulds. These have been called higher bacteria (trichobacteria?) (see table, p. 26). They show increased complexity of structure and function: (1) in forming irregularly segmented filaments that under certain conditions usually produce branches; (2) in developing certain portions of their substance into reproductive bodies from which the new individuals grow (Plate I, Figs. 15 and 16).

The filaments seen sometimes among the lower forms have independent segments, which may easily separate and grow as tiny unicellular forms, while in the higher forms the filaments in their growth show a certain interdependence of their parts. For example, growth often occurs from only one end of the filament while the other becomes attached to some fixed object. These latter are all saprophytes.

The members of the higher bacteria which are pathogenic have been incompletely studied and classified as yet.

[1] Beiträge zur Kenntnis der Bakterien, etc., Arch. f. Protistenk, 1902, **1**, 306, and 1903, **2**, 416.

Foulerton[1] and his associates made an extensive study of this group, both saprophytic and parasitic varieties, and they agreed with some others in calling attention to the acid-fast character of some of the varieties and to the apparent relationship of the group to B. tuberculosis, B. mallei, and B. diphtheriæ. To us, however, the relationship does not seem to be close enough to place all of these organisms in one group. We show that the apparent branching in B. diphtheriæ is not a true branching.

Foulerton considers all organisms in the group classed as higher bacteria as belonging to a single genus, streptothrix, which he places with the hyphomycetes, or mould fungi, because of their growth in branching threads from spore-like bodies. He says that streptothrix and actinomyces are absolutely synonymous terms, and that the majority of pathologists consider them so.

Wright[2] and others called attention to the fact that the term "streptothrix" had been used already for a mould genus, and Wright recommended reviving the term "nocardia" for those branching forms that did not produce the ray-shaped groups. But since MacCallum and others have shown that most, if not all, the forms called streptothrix or nocardia may produce ray-like growths under suitable conditions it is recommended[3] that, until further study, all of these forms should be called actinomyces. This genus therefore contains a large number of species, only a few of which are pathogenic.

Some of these species have varying degrees of acid-fastness, some have none. Most are Gram-positive, some are Gram-amphophile and a few are Gram-negative. Some are anaërobic, some are microacrophilic and many are aërobic. So there seem to be enough characteristics to make several genera. Bergey has collected them into three groups—those pathogenic for man and lower animals, those pathogenic for plants and those that are saprophytes (of which Waksman has reported many).

The earlier genus Cladothrix has been given up because the descriptions given of the forms placed under it are all too meager to exclude them from being actinomyces. Two new genera are added to this group, hence, according to the Society of American Bacteriologists, we have the following four genera in the family.

1. *Actinobacillus.*—Filaments formed in cultures. In lesions finger-shaped branched forms are seen.

2. *Leptotrichia* grows stiff, almost straight threads, in which division processes are seldom or never observed, and no branching has been seen.

3. *Actinomyces* grows in threads with true branching. Spores or conidiæ have been observed. It is characterized by the radiating, wreath-like forms which it produces in the living body.

4. *Erysipelothrix* forms rod-shaped organisms with only an occasional long filament which may show slight branches.

Reproduction among the Higher Bacteria.—These forms increase in length for a time and then, at the free ends, or at intervals along the filaments, they may produce small rounded cells, called conidia or spores, from which new individuals are formed. The terminal spores in some genera may be flagellated after their separation from the parent filament.

[1] Lancet, 1910, **178**, 551, 626 and 769.

[2] Jour. Exp. Med., 1898, **3**, 421; Jour. Med. Res., 1905, **8**, 349, and Osler's Modern Medicine, 1907, **1**, 327.

[3] Recommendations of Society of American Bacteriologists committee and Bergey.

YEASTS AND MOULDS (EUMYCETES).

According to the latest classification, the microbes commonly known as yeasts and moulds are so intimately related that they must be considered together. They may be described in general as being much larger on the whole than bacteria, more markedly filamentous, and often multicellular. They multiply by budding and by sexual and asexual spores. Those that grow in branching filaments or threads (hyphæ) forming a mycelium are called moulds. Those that grow usually as rounded cells that multiply chiefly by budding are called yeasts.

Based upon the ability of certain of these fungi to form septate mycelium in the vegetative stage and to form asci (sacs containing spores) in fruiting stage, they have been divided into groups or classes (see Table, p. 26).[1] The forms pathogenic in man fall into three of these classes. Among members of the first class—the fungi imperfecti—the mycelium if present is septate, and no asci are seen. ' Members of the second class—the ascomycetes—have septate mycelium and they produce asci, generally containing four spores or a multiple of this number; members of the third class—the phycomycetes—form no septate mycelium in the vegetative stage, neither do they form asci.

Among the fungi imperfecti are found many species pathogenic for man. By consulting the table it is seen that some forms commonly called yeasts are placed in this class and the others are classed with the ascomycetes. The third class has only one genus containing species pathogenic to man—the genus Mucor. As an illustration of the importance of molds to man, Thom[2] has given a description of one group of the ascomycetes, the aspergilli.

The most important property of yeasts, though one not possessed by all to the same degree, is that of producing alcoholic fermentation. The enzyme causing this action is called zymase. In brewing we distinguish between the yeasts that can be employed practically, "culture

EXPLANATION OF PLATE III.

Partly schematic. Rearranged and drawn by Williams from the indicated authors.

Fig. 1.—Aspergillus glaucus. Fruiting hyphæ growing from mycelium: *A*, conidiophore; *B*, sterigma; *C*, conidia; *D*, beginning perithecium; *E*, conidiophore bearing spores; *F*, perithecium containing rudiments in section; *G*, ascus containing eight spores (De Bary).

Fig. 2.—Penicillium, showing formation of conidia, *A*.

Fig. 3.—Mucor mucedo: *A*, sporangium containing spores; *B*, spores liberated; *C*, chlamydospores; *D*, *E*, *F*, stages in the formation of a zygospore.

Fig. 4.—Oïdium lactis.

Fig. 5.—Sporotrichum schenkii, showing formation of whorled spores on branched mycelium.

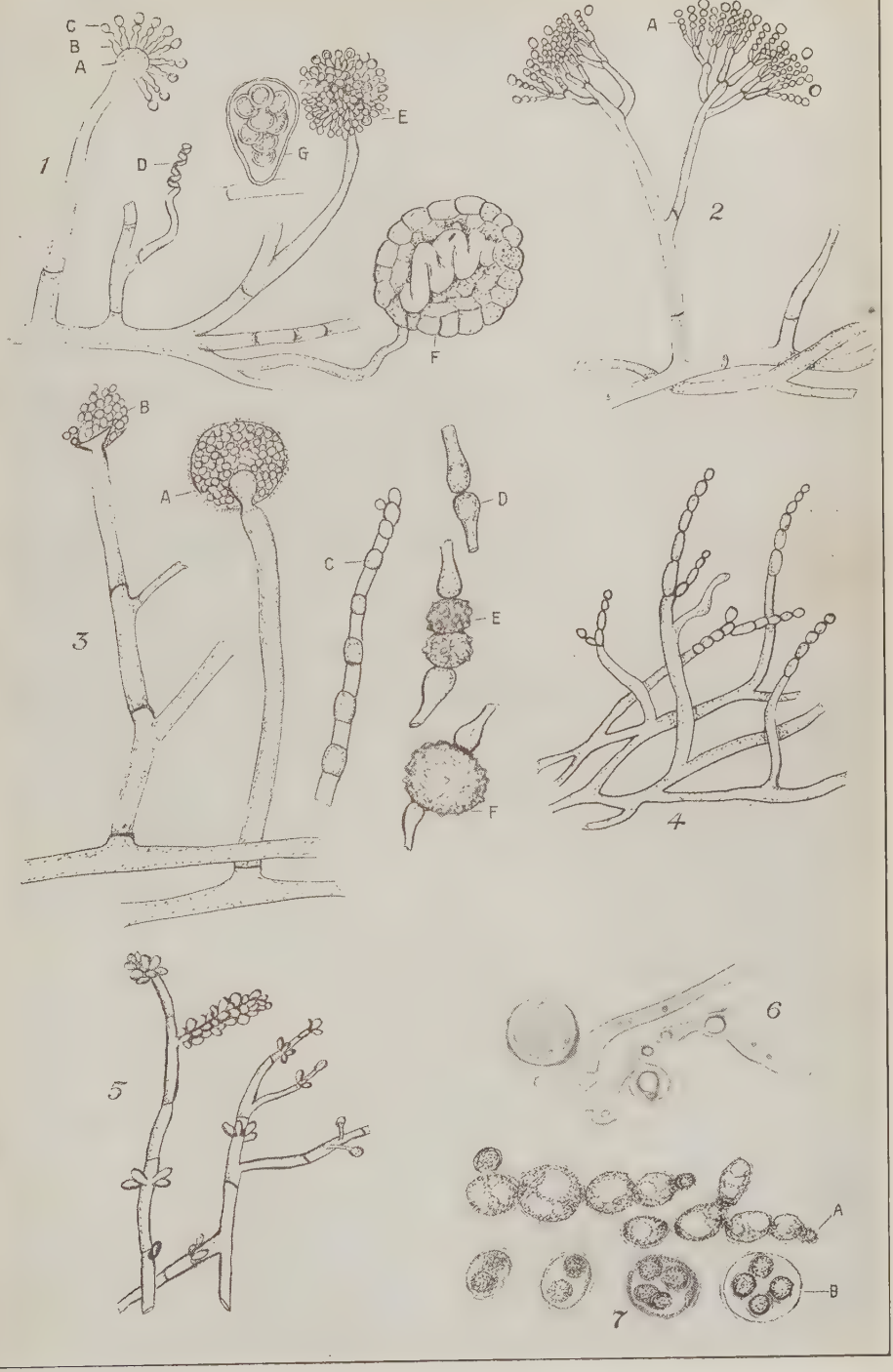
Fig. 6.—Yeast from human infection in culture showing mycelium-like growth.

Fig. 7.—Saccharomyces cerevisiæ (Hansen): *A*, budding; *B*, spore formation.

[1] Castellani: Fungi and Fungous Diseases, Jour. Am. Med. Assn., 1928.

[2] Jordan and Falk: The Aspergilli, in Newer Knowledge, Chicago, 1928.

PLATE III



yeasts," and those which often act as disturbing factors, so-called "wild" yeasts.

The shape of most of the culture yeasts is oval or elliptic (Plate III, Fig. 7). Round or globular forms are more often met with among the wild species which usually excite only a slight degree of fermentation. But sausage-shaped and thread forms are also met with. Pathogenic forms may also be round.

The individual yeast cells are strongly refractive, so that under the microscope at times they have almost the luster of fat droplets. This is important because in examining fresh tissues the yeast cells may be hard to distinguish from fat droplets, often requiring the aid of certain reagents for their identification.

The size of the individual yeast cells varies enormously, even in those of the same species or the same culture. In old colonies individuals may be found no larger than cocci, 0.5μ to 1μ in diameter, while in other colonies, especially on the surface of a liquefied medium, giant yeast cells are found often attaining a diameter of 40μ or more. In spite of these wide fluctuations, however, the various species are characterized by a fairly definite average in size and form. Each cell contains a more or less definite nucleus, which is demonstrated by the usual chromogenic stains.

During the process of budding the nucleus of the cell moves toward the margin, where it divides. At this point the limiting membrane of the cell ruptures or usually a hernia-like protrusion develops which has the appearance of a button attached to the cell. The daughter-cell so formed rapidly increases in size and gradually assumes the shape and size of the mother-cell.

A fact of the utmost importance for the propagation of the blastomycetes and continuation of the species is the formation of spores which may take place either by the changing of special cells as a whole into single spores or by the dividing of the nuclear material of a cell into several fragments, each of which becomes the center of a new cell lying within the original cell, or ascus (Plate III, Fig. 7). These new cells possess a firm membrane, a cell nucleus and a little dense protoplasm. The number of these endospores developed in the yeast cells varies, but is constant for a given species. As a rule, one cell does not produce more than four *endogenous* spores, but species have been observed, *e. g.*, *Schizosaccharomyces octosporus* (Beijerinck), in which eight spores are found. Guilliermond has described conjugation in yeasts before the formation of spores.

Yeasts stain readily in the young state with ordinary dyes. They are Gram-positive. Older forms stain very irregularly. They are cultivated—sometimes with difficulty directly from tissue—on ordinary media, best on media made slightly acid (see chapter on Media). Some pathogenic varieties grow best at blood heat. Most varieties grow easily at room temperature.

The vitality of yeasts and moulds is truly enormous. Growths may be obtained from spores dried for over a dozen years.

PROTOZOA.

Definition.—A protozoön (the lowest form of life classed in the animal kingdom) is a morphologically single-celled organism (Dobell prefers calling it a non-celled organism), composed of protoplasm which is differentiated into cytoplasm and nucleus (or nuclear substance), both of which show many variations throughout the more or less complicated life cycle that each individual undergoes.

The protozoa are of higher grade than the bacteria because of their greater complexity in structure and life cycle (Plate IV).

Their shape and size vary so widely that no general description will fit all types. Some forms are small enough to pass through a Berkefeld filter, while the largest varieties described are about $\frac{2}{3}$ inch long.

The Cytoplasm.—The cytoplasm consists of a mixture of substances, the most important of which belong to the proteins. It is more or less fluid, but, because of differences in the density and solubility of the several parts, it often presents an alveolar, linear, or granular appearance, which may come out clearly in fixed and stained specimens, but is usually not well seen in the living cells.

Ectoplasm and Entoplasm.—Frequently the protozoön cytoplasm is differentiated into a concentrated, viscid, more homogeneous, or hyaline outer layer called the *ectoplasm* and a more fluid granular central portion called the *entoplasm*. These two portions have different functions. The ectoplasm helps form the various organs (organelles) of motion, contraction and prehension such as pseudopods (false feet), flagella (whip-like threads), cilia (hair filaments), suctorial tubules (through which food passes) and myonemes (contractile fibrils found in ciliates, gregarines and a few flagellates). The entoplasm digests the food and contains the nucleus, as well as various granules and vacuoles. Some vacuoles contain fluids that serve as food digesters—digestive ferments. The so-called contractile vacuoles which periodically fill and empty themselves may be considered as excretory organelles.

Other substances are seen from time to time in the entoplasm, such as bacteria, red blood cells, fatty granular pigments, bubbles of gas, crystals, etc.

The Nucleus.—The simplest morphological nucleus is a vesicular body which is differentiated from the cytoplasm by an essential constituent, chromatin, so called because it has a strong affinity for certain basic staining materials. Chromatin consists mostly of nuclein and appears in the form of smaller or larger granules, masses, or rods.

Generally, the chromatin particles are mixed with a second less intensely staining substance with more of an affinity for acid stains called plastin or paranuclein, similar to the substance from which the true nucleolus of the metazoön cell seems to be formed. This substance may appear in one or more distinct, rounded bodies. Most of the chromatic substances of the nucleus in many protozoa are often massed together in an intensely staining ball-like body called the *karyosome* which undergoes various cyclic changes during the growth and develop-

ment of the organism. The centrosome is generally imbedded in the karyosome. The chromatin and plastin lie imbedded in a third substance in the form of an achromatic network called linin, which is closely related to the cytoplasmic network. There may or may not be a definite nuclear membrane. Sometimes there is no definitely structured nucleus, but the nuclear substance in the form of small chromatin masses or granules is distributed throughout the cytoplasm (the so-called "distributed nucleus" similar to that seen in bacteria).

Somatic and Generative Chromatin.—Some chromatin substances of the cell have physiological properties different from others. At times substances which have only vegetative properties are active, forming the so-called somatic or trophic chromatin; at other times, substances appear during sexual activities called generative or sexual or idiochromatin, and from these the vegetative (somatic) chromatin for the new cells is again formed. In the ciliata both these chromatin elements are present as distinct morphological bodies during the entire life of the organism, the somatic form in the macronucleus and the generative form in the micronucleus.

Chromidia.—The chromatin elements, in the form of granules, small irregular masses, threads, network, etc., which at certain stages pass from the nucleus into the cytoplasm, or which at times are, possibly, formed in the cytoplasm, were named "Chromidien" by R. Hertwig, who in 1899 first described their appearance. Their function in generative processes was demonstrated in 1903 by Schaudinn. During their formation the nucleus may entirely disappear, so that morphologically the cell may be considered non-nuclear. At a definite time thereafter new typical nuclei may be formed from these chromidial substances.

Locomotor Nucleus (*Kinetic Nucleus*).—In flagellates still another definite physiological chromatin is seen in the small body called the kinetic nucleus, which is either apart from or merged into a smaller body, the blepharoplast, forming the root of the flagellum. The kinetic nucleus is so called because it produces the locomotor apparatus. Both the kinetic and trophic nuclei may contain somatic and generative chromatin at the same time.

The Centrosome.—This is a small body which is always present in metazoan cells, playing an important part in cell division, but it has not been demonstrated as a morphological entity in many varieties of protozoa; part of the karyosome, however, may take its place, or there may always be a true centrosome within the karyosome. Whenever a centrosome appears in protozoa, it has its origin in the nucleus, resembling in this the kinetic nucleus and blepharoplast. All these four bodies (centrosome, blepharoplast, kinetic nucleus and karyosome) therefore may be considered as having a similar morphological origin.

Physiological Characteristics of Protozoa.—In common with all other living organisms protozoa possess the characteristics of motility, nutrition, respiration, and reproduction.

Motility.—All protozoa react in certain characteristic ways toward chemical, mechanical, and electrical stimuli. Many are affected by light, while probably none reacts to sound. They manifest the reaction usually by motion of some sort. Most animal parasites, especially the higher forms, exert a positive taxis for leukocytes, principally for the large mononuclears and the eosinophiles. This fact is made use of in clinical diagnosis.

Nutrition.—Many protozoa, especially the pathogenic forms, absorb fluid food directly through the body wall; but the majority take in solid food, such as small animal or vegetable organisms and organic waste, some through more or less definite regions of the body, others through any part of the surface by extending pseudopodia and entirely surrounding the food object, forming a so-called gastric vacuole.

After the food is digested the waste products are excreted, sometimes by osmosis, generally through special structures as the contractile vacuoles which regularly eject fluid substances to the outside of the organism.

Respiration.—It is supposed that the contractile vacuole has a respiratory as well as an excretory function. The interchange of gases is always going on, if not through a contractile vacuole, then by osmosis through any part of the wall.

Growth and Reproduction.—Under favorable conditions, new protoplasm is constructed rapidly, and the mass increases faster than the surface which, according to Spencer, initiates cell division. The changes generally appear first in the nucleus. The simplest variety of reproduction is a two-celled fission which may be either longitudinal or transverse, either of which may be direct (amitotic) or indirect (mitotic). A modification of equal fission is the so-called budding which may be single or multiple. When growth occurs so that fission is for a time incomplete, one cytoplasm containing several nuclei which finally separate into as many daughter organisms, the process is called multiplicative reproduction, or brood formation or internal budding. In the most extreme cases of multiplicative reproduction as it occurs among sporozoa, the mother-cell with its nucleus separates simultaneously into large numbers of tiny daughter-cells. Such a process, when it occurs without conjugation and encystment, is called schizogony and the new cells are called merozoites. When such a multiplicative division occurs (generally after fertilization) within a cyst, it is spoken of as sporogeny and the new cells are called sporozoites.

Sexual Phenomena.—Sexual phenomena (syngamy) fundamentally similar to those seen in metazoa have been observed in all groups of protozoa studied. The reproduction by the usual division or budding is interrupted at certain times in the life history of each organism and individuals come together in such a way that their nuclei fuse, usually after having undergone characteristic reduction divisions.

When the union is permanent, we speak of it as copulation and liken the process to that of the fecundation of the ovum by a spermatozoön. When the union is transient we call it conjugation. Here the two cells fuse for a time when the nuclei interchange protoplasm and then the cells separate and each one continues to grow and divide independently. When in a partly divided cell or in an apparently single cell, two nuclei, after undergoing reduction division, or its like, fuse, the process is called autogamy.

The developmental cycle of a protozoön consists of all the changes which occur in its growth from one act of fertilization to another (Plate

IV, Fig. III). Many protozoa carry on the sexual part of their life cycle in one host and the asexual part in another (*e. g.*, malarial organisms).

Cyst Formation.—If protozoa do not get the required amount of water or air or suitable food, they cease their special movements, round out into more or less of a sphere and form a resisting membrane of chitin within which they may live for a long time, withstanding periods of desiccation, extreme heat and cold, and they may be blown about as dust until they find conditions again favorable for renewed growth; then water is absorbed, the cyst is ruptured and active life begins anew.

In parasitic forms encystment plays an important part in the passage from the old host to the new. The majority of forms would not be able to exist outside of the body of the host without having some protective membrane. The cyst may be formed simply for protection from drought, etc., when it is called a hypnocyst, from which the organism may emerge in about the same form as when it encysted; or the cyst may precede reproduction by spore formation or simple division, when it is called a sporocyst. In either case it may consist of a simple wall or it may be formed of several walls to enable it to resist prolonged desiccation, when it is called a resting cyst.

Characteristics of Each of the Four Groups of Protozoa.—Flagellata.—Flagellata are protozoa which move in the adult forms by one to several flagella or whip-like processes. If pseudopodia develop, they are transitory.

Generally the flagella arise from the anterior part of the organism, and in motion the larger ones (primary flagella) are directed forward, while smaller ones (secondary flagella) are directed backward, acting as rudders. Certain flagellata possess a modification of their bodies in what is called the undulating membrane, which consists of a fluted protoplasmic process attached along one side of the organism, the free edge of which is prolonged as the flagellum. It has been shown that flagella are not simple protoplasmic processes, but that they have more or less of a framework of elastic fibers as well, hence their power in locomotion can be better understood. Except with special stains, which bring out these fibers, the flagella appear homogeneous.

The flagella arise from some definite place in the cytoplasm, sometimes from a distinctly differentiated chromatic body which has been given various names, such as blepharoplast, kinetic nucleus or centrosome, sometimes near this from a basal granule, microsome, diplosome, or flagellum root, sometimes directly from the nucleus. The basal granules seem to be derived primarily from the kinetonucleus, and may be considered from a physiological standpoint as a part of the motor nuclei.

The body of the flagellates is generally more or less elongated and, except in most primitive ones, is fixed in its outline. The latter characteristic is chiefly due to the fact that the organisms usually possess definite though delicate membranes containing elastic fibrils. The cytoplasm is usually not differentiated into an ento- and ectoplasm. It often contains one to several contractile vacuoles, as well as food

vacuoles, and there is frequently a definite opening or cytostome for the entrance of food. There are usually many granules and inclusions of various kinds scattered throughout the cytoplasm, and myoneme striations are seen in some forms. The nucleus, as a rule, situated anteriorly, varies much according to different species and to different stages of development.

EXPLANATION OF PLATE IV.

Partly schematic. Rearranged and drawn by Williams. All stained by Giemsa.

I. FLAGELLATES.

FIG. 1.—Illustrating one flagellum. Leishmania: *A*, intracellular forms; *B*, cultural forms.

Fig. 2.—Illustrating undulating membranes: *A*, Trypanosoma lewisi; *B*, Trypanosoma brucei; *C*, Trypanosoma gambiense.

FIG. 3.—Illustrating two flagella. Bode lacertæ (after Prowazek).

FIG. 4.—Illustrating four flagella. Trichomonas.

II. AMEBÆ.

Illustrating points considered differential in the two common types of amebæ (endamebæ) described as parasitic in human beings.

FIG. 1.—Endameba coli, vegetative stage.

FIG. 2.—Dividing nucleus.

FIG. 3.—Endameba coli cyst containing eight nuclei.

FIG. 4.—Endameba histolytica, vegetative stage.

FIG. 5.—Four-nucleate cyst.

III. SPOROZOA.

A, description of Figs. 1 to 16. (After Schaudinn.) The life cycle of Eimeria schubergi. In 1, the sporozoites, becoming free by bursting the sporocysts, pass out through an aperture in the wall of the oöcyst, and are ready to enter the epithelial cells of the host. 2 to 6 represent the asexual reproduction or schizogony, commencing with infection of an epithelial cell by a merozoite or a sporozoite; the merozoite after stage 6 may start again (5) at stage 2, as indicated by the arrows, or it may go on to the formation of gametocytes (9 to 11). 9 to 11 represent the sexual generation, the line of development becoming split into two lines—male (♂) and female (♀)—culminating in the highly differentiated gametes, which conjugate and become again a single line, shown in 12–14. The zygote thus formed goes on to the production of spores, 15 and 16. 2 and 3 represent epithelial cells showing penetration of a merozoite or a sporozoite and its change into a schizont; 4, the nucleus of the schizont divided into numerous daughter-nuclei; 6, segmentation of the schizont into numerous merozoites, about a central mass of residual protoplasm, which in this figure is hidden by the merozoites; 5, merozoites passing to reinfect host cell and repeat the process of schizogony; 7, 8, merozoites to be differentiated into male and female gametocytes; 9, the two gametocytes within a host cell (the microgametocyte (♂) has fine granulations; the macrogametocyte (♀) has coarse granulations); 11, a female gametocyte undergoing maturation; 13, mature macrogamete, free from the host cell, and sending a cone of reception toward an approaching microgamete. In 12 the nuclei of the last stage have become microgametes, each with two flagella. The free microgametes are swimming to find a macrogamete. 14, the zygote (fertilized macrogamete), surrounded by a tough membrane or oöcyst, which allows no more microgametes to enter and containing the female chromatin, which is taking the form of a spindle, and the male chromatin in a compact lump; 15, the nucleus of the zygote divided—the nuclei of the sporoblasts; in 16 the four sporoblasts become distinct, leaving a small quantity of residual protoplasm; each sporoblast has formed a membrane, the sporocyst. Within each sporocyst two sporozoites form about a sporal residuum.

B, Babesia infecting red blood cells: 1, pear-shaped bodies; 2, dividing forms; 3, eight pear-shaped bodies in a cell; 4, irregular ring-like bodies; 5, large, irregular body; 6, body with a flagellum-like projection.

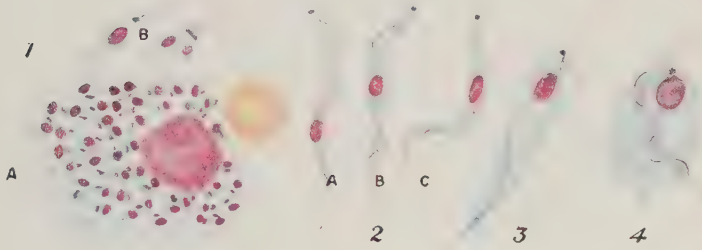
IV. CILIATES.

FIG. 1.—Balantidium coli (after Hartmann): *A*, adult form; *B*, *C*, dividing forms; *D*, conjugating forms.

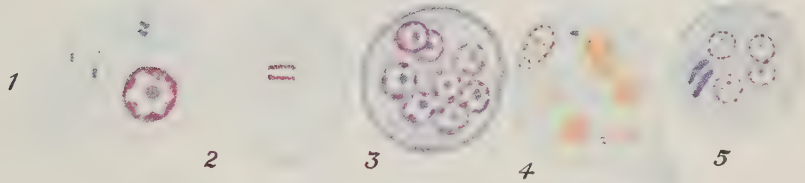
PLATE IV

TYPES OF PROTOZOA

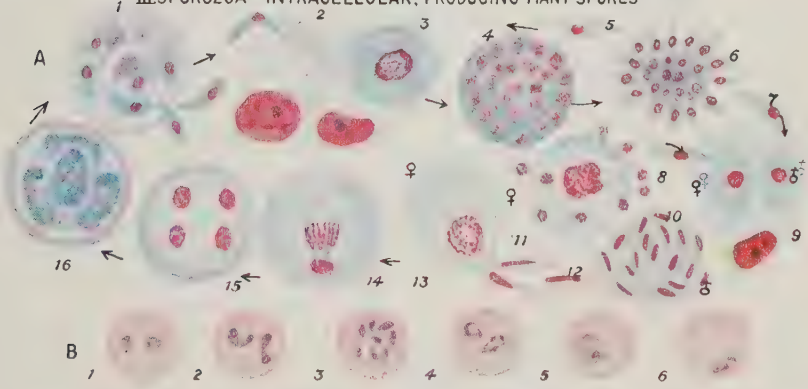
I. FLAGELLATES – MOVING BY FLAGELLA



II. AMEBÆ – MOVING BY PSEUDOPODS



III. SPOROZOA – INTRACELLULAR, PRODUCING MANY SPORES



IV. CILIATES – MOVING BY CILIA

A. W. WILLIAMS, DEL.

The flagellata multiply either in the free motile condition or after encystment. In the first case, as a general thing, they divide longitudinally. The basal granules divide with the nuclei and the flagella of the daughter organisms are usually formed anew. Multiple division is also observed. In the second case the flagellata may or may not conjugate before they encyst. Then they divide within the cyst.

The sexual cycle varies much in different species. Isogamy has been noticed between fully grown individuals as well as between smaller forms. The union of different-sized forms, or anisogamy, has also been observed. Also autogamy is said not to be infrequent. It is claimed that certain of the flagellates pathogenic for man require a second host for the development of their sexual cycle.

The different types of the chief pathogenic flagellates are shown in Plate IV, 1, Figs. 1 to 4. Among the most important pathogenic forms are the trypanosomes.

Trypanosoma.—The name trypanosoma (boring animal) was given by Gruby, in 1843, to certain free-swimming hemoflagellates found by him in the blood of frogs. Much later similar flagellates were found in the blood plasma of many different species of vertebrates and in the intestinal tract of several blood-sucking invertebrates. Some of the forms, including those found in man, are pathogenic. A number of the blood-sucking insects are carriers of the diseased species to healthy animals. There has been an attempt recently to split this genus into several genera—Salmonella, Duttonella, Lewisonella—but this division has not been generally adopted.

Typical trypanosomes are characterized by a comparatively long, spirally twisted body, along one side of which is attached an undulating membrane having a cord-like edge that is continued forward as a free whip (flagellum). The flagellum arises near the posterior end of the organism in a small granule called the basal granule, which may be connected with the blepharoplast, a larger chromatin mass, called also the kinetonucleus because of its control over the motor apparatus. The nuclear apparatus consists of a macro- or trophonucleus, and of the above-mentioned kinetonucleus or blepharoplast, which last functions as a centrosome. The trophonucleus is usually situated near the middle of the organism; it is granular, thick and egg-shaped, but varies somewhat in size and shape. The cytoplasm is faintly alveolar or granular, varying with age, environment and possibly species. Toward the straight border of the cell the cytoplasm is more or less striated and in a few species definite myonemes are seen.

Reproduction occurs usually by longitudinal, occasionally by multiple division. The life cycle is not well known. Though transmission occurs through the bites of various invertebrates, notably flies, the few sexual changes described as taking place in the intestines of some of these intermediate hosts have not been fully corroborated. That an intermediate host is not necessary for the continued life of at least one species of trypanosome seems to be proved by the fact of direct transmission of T. equiperdum from horse to horse through coitus.

Leishmania.—Another important pathogenic flagellate genus is Leishmania. In humans this grows chiefly within large mononuclear cells. It shows its flagellated forms in cultures. (Plate IV, Fig. 1. See also Part II.)

Amœbæ.—Under amœbæ (Family, amœbidæ) we include forms composed of naked, simply constructed protoplasm having the power of producing lobose pseudopodia which are used as organs of motion and of nutrition.

The pseudopodia are protoplasmic processes which are projected in irregular succession from different parts of the surface of the cell, producing in this way an irregular motion. The form of the pseudopodia varies considerably in the different species. For instance, there are broad, blunt processes or narrow, less blunted ones, and each may be short or long, single or slightly branched. The cytoplasm may or may not take a share in their formation. The forms, of course, vary within limits according to the condition of the medium in which the amœbæ are living. Movements are always called forth by some physical or chemical excitant. When such an excitant is desirable for food the pseudopods flow around it, and it is subsequently absorbed in the cytoplasm of the organism.

The members of this group may possess one nucleus or several. *Amœba binucleata* has two nuclei in the young adult stage, and *Pelomyxa palustris*, living in the bottom ooze of ponds, has an enormous number of nuclei. A marked feature of the nuclear apparatus is the formation of chromidia which, as has already been noted, may play such an important part in sexual reproduction. Generally each amœba at some stage of development has one contractile vacuole, but occasionally some are seen with several or with none.

Saprozoic forms belonging to this order are common. They may be found wherever there are moisture and decaying vegetable matter. The pathogenic forms are not so frequent.

Notwithstanding the common occurrence of saprozoic forms, the full life history of few of them has been worked out, and until the full cycle of development of any so-called amœba is known it is impossible to say whether that particular form belongs among rhizopoda or whether it is a developmental form of another group, as amœboid forms may occur at some time in the life history of all groups. It is quite possible that some of the organisms described as belonging to this order are really members of entirely different orders. For instance, it is known that the flagellate *Trichomonas* loses its flagella before copulation and crawls about by means of short blunt pseudopods as a typical amœba.

Amœbæ reproduce by simple fission, by budding, and by brood formation. In the last case the reproduction is usually preceded by encystment. Two forms have been described as most frequently parasitic in man. The chief differences claimed to exist between them are shown in Plate IV, II, Figs. 1 to 5.

The Sporozoa.—The sporozoa are a group of exclusively parasitic protozoa of very widespread occurrence, living in the cells, tissues, and

cavities of animals of every class. Generally they are harmless, but some varieties may produce pathological changes and even fatal diseases severely epidemic.

As their name indicates, they are all characterized by reproduction through spore formation, but they exhibit the utmost diversity of structural and developmental characteristics. As a rule, each species is parasitic on one kind of tissue of a particular species of host. They are generally taken into the system in the spore stage either (1) with the food of the host, (2) by the bites of insects, or (3) by inhalation. The spore membranes are dissolved by the fluids of the host, and thus one or more germs or sporozoites are set free to bore into the special cells of the host. Here they grow, some remaining permanently intracellular, others only in the young stages. The latter either pass different phases of their more or less complicated life history in different parts of the body of one and the same host or they pass some phases of their life cycle in the cells of an intermediate host.

The sporozoa vary widely in size as well as in other characteristics. From the smallest, several of which can be contained in a single blood cell, there are all gradations in size up to those that may be seen by the naked eye (*Porospora gigantea*, 16 mm.).

Besides being characterized by the power to produce very many resisting spores, the sporozoa are also characterized by the fact that as a class they possess none of the special organs found in other protozoa for ingesting or digesting solids. Many develop flagella during sexual phases or show amœboid movement during certain stages of their life cycle, but the flagella and pseudopodia are organs of locomotion and not of nutrition. Food vacuoles or contractile vacuoles have not been found. The life cycle of a typical sporozoön is rearranged and condensed from Schaudinn in Plate IV, III, Figs. 1 to 16.

Infusoria.—The infusoria (Plate IV) belong to the most complex of the protozoa. They possess a definite entoplasm containing nuclei and food vacuoles, and a definite ectoplasm containing basal granules from which arise the cilia which give the group its name. They have organoid structures which receive the food, some having definite mouth openings and definite places for excreting waste products. The food vacuoles may contain acid or alkaline digestive products. The nuclear material is differentiated into two forms, a large macronucleus and a much smaller micronucleus. The function of the macronucleus is supposed to be vegetative, and that of the micronucleus reproductive. The macronucleus varies in size and shape and is completely filled with an alveolar chromatin. The micronucleus also varies in size and shape, and, except in reproductive phases, is generally vesicular in structure, with the chromatin heaped in one mass. Division of the nuclei takes place by mitosis in the case of micronuclei, and by amitosis, as a rule, in the case of the macronuclei. Under conditions unfavorable for growth the ciliata may encyst.

Conjugation seems to be necessary to the life activity of these organisms. The phenomenon of conjugation in the ciliata has been well

worked out. The micronuclei play the most important part, whereas the macronuclei simply break up and disappear in the protoplasm.

According to the arrangement of the cilia, the ciliata are divided into the four orders given in the general classification (see p. 26). Among these, the second, the order of the Heterotricha, interests us. In the Heterotricha the cilia are uniform over most of the body, while a specialized set fused into a series of firm vibratory plates is found about the mouth. Only one genus, *Balantidium*, is said to be pathogenic in man. (Plate IV; also Part II).

CHEMICAL COMPOSITION OF MICROÖRGANISMS.

Quantitatively considered, the bodies of microörganisms consist largely of water (bacteria from 80 to 90 per cent.). The other constituents are: albuminous substances (about 10 per cent.), containing nitrogen, oxygen, hydrogen, carbon salts (chiefly phosphorus, potassium, chlorine, calcium, iron, magnesium and sulphur), fats[1] and in smaller quantities, extractive substances soluble in alcohol and in ether. Special varieties contain unusual substances, as wax and hemicellulose in tubercle bacilli. Each variety yields protein substances peculiar to itself, as shown in the effects produced by animal inoculation. At present we know but little concerning the differentiation of these specific substances. This subject will be taken up in detail under Toxins, etc. According to Cramer,[2] many bacteria contain amyloid substances which give a blue reaction with iodine. True cellulose has not been found in bacteria by Vaughan[3] or other workers, but large quantities of a gelatinous carbohydrate similar to hemicellulose have been obtained. The nuclein bases—zanthin, guanin and adenin—have been obtained in considerable amounts. Nuclein is found in all microorganisms. Nucleic acid has been obtained from yeasts and from the few bacteria tested. Recently it has been obtained pure from Escherichia communior.[4] The question as to whether bacterial nucleic acids are an entity different from either plant or animal nucleic acid[5] is still being studied. Vaughan found no sodium chloride in his alcoholic extracts. There is a group of bacteria which contains large amounts of sulphur—viz., the *Beggiatoa*—and another group, the *Cladothrix*, is capable of separating ferric oxide from water containing iron.

Microörganisms possess the capacity to a high degree of accommodating their chemical composition quantitively to the variety of soil in which they are growing. Thus, *B. prodigiosus*, when grown on potato, contains 21.5 per cent. of dry residue and 2.7 per cent. of ash; when cultivated on turnips it contains 12.6 per cent. of dry residue and 1.3 per cent. of ash. Besides the concentration of the culture, its temperature and age also influence the amount of residue and ash produced.

[1] Larson and Larson (with Bibliography): Jour. Inf. Dis., 1922, **31**, 407.
[2] Arch. of Hygiene, vols. **12** to **28**. [3] Herter Lectures. 1915
[4] Schaffer and Beyn Jones: Bull. Johns Hopkins Hosp., 1922, **33**, 151.
[5] Jones, Walter: The Nucleic Acids, New York, 1914.

Qualitatively, a variation is shown by the addition of peptone in the culture media which tends to increase the percentage of nitrogenous matter in the microbe, or by the addition of glucose which decreases it.[1]

The chemical composition of the bodies of animal parasites is an almost unexplored field. The ectoplasm and the cyst sacs in general are made up principally of a substance called chitin. Glycogen has been isolated from many forms. Proteolytic enzymes and acid secretion in digestive vacuoles have been demonstrated.

The Vitamin Content of Microörganisms.—A number of investigations have been made recently to show that certain microörganisms (notably yeasts) may contain vitamins. Just what relation these substances have to the "bios"[2] of Wildiers, and to the vitamins shown to be necessary for the growth of animals is undecided. It has also not been shown whether they are an essential part of the organism or are a variable factor dependent upon the kind of culture medium used.[3]

Microchemical Reactions.—To a certain degree the chemical composition of the *individual* organism may be studied both in the living and in the dead individual by the addition of the testing substances to a hanging drop or to a spread of such organism and the examination of it under the microscope. Thus, fats have been demonstrated by staining with osmic acid, Sudan III, or Scharlach R., as well as by alcohol-ether extraction.

Of special importance in this regard is the resistance which bacteria possess to diluted alkalies. Inasmuch as the majority of animal tissues are dissolved when treated with alkalies, this method has been adopted for rendering visible unstained bacteria in tissues. (See also Principles of Staining, p. 78.)

EFFECTS OF SURROUNDING FORCES UPON MICROÖRGANISMS.

Food.—Though the majority of pathogenic microörganisms grow easily on certain artificial foods (culture media), some of them, like many of the protozoa, we have not yet been able to cultivate outside of the body of their host. Those microörganisms which seem to depend entirely upon a living host for their existence are known as *pure parasites*; those which live only upon dead organic (a few on inorganic) substances are called *pure saprophytes*, if plants, or pure saprozoa if animals; those which can lead a saprophytic or saprozoic existence, but which usually thrive only within tissues, are called *facultative saprophytes*, while those that grow usually on dead material, but may grow within living tissues, are called *facultative parasites*. The separation between these groups is not sharp. The *saprophytic* or *saprozoic* forms which represent the large majority of all microörganisms are not only harmless

[1] Baumgärtel: The Chemical Structure of Bacteria, Newer Knowledge, Chicago Press, 1928, p. 14.

[2] Wildiers: La Cellule, 1901, **18**, 259.

[3] Williams and Olsen: Jour. Biol. Chem., 1923, **55**, 815. Many investigations on this subject have been published in this journal and others during the last few years.

to living organisms, but perform many exceedingly important functions in nature, such as the destruction of dead organic matter and its preparation for plant food through decomposition, putrefaction and fermentation, while one group (see below, the Nitrifying Bacteria) are constructive in their activities. The *parasites*, on the contrary, may be harmful invaders (pathogenic microörganisms) of the body tissues, exciting by their growth and products many forms of disease. (See chapter on Relation of Microörganisms to Disease.)

The substances essential for the majority of those forms which can be grown artificially are: organic compounds as a source of carbon and nitrogen, an abundance of water and salts containing the essential elements already listed. The question as to the form of nitrogenous compounds that is best for growth has been studied by Rettger and his co-workers,[1] and several others. They state that native proteins are not directly utilized by bacteria. They studied also[2] the amino-acids and related simple nitrogenous compounds, and found that while many saprophytes were able to use these substances as a source of nitrogen, few of the pathogens studied showed growth. Studies on the metabolism of bacteria have been summarized by Kendall,[3] who with his associates, have made many studies on this subject. The polypeptid N fraction seems to be chiefly altered in bacterial metabolism.

Growth Accessory Substances.—The question as to the nature of those substances that promote the growth of microörganisms has received an impetus in these years through the renewed study of the factors that cause the growth of the "influenza group" of bacteria,[4] and the work of the physiological chemists on vitamins. There is no question but that substances of a vitamin nature as well as other special substances help the growth of microörganisms, as they do that of higher beings. It has been shown that certain microörganisms cannot grow without very special substances. Thus, the hemoglobinophilic bacilli seem to require two substances for their growth—a "V factor" which is a vitamin-like substance and an X factor (see chapter on Influenza Bacillus).

Reaction of Media.—The reaction of the media as to acidity or alkalinity is of very great importance. Most bacteria, particularly the pathogenic forms grow best on those media that approach neutrality. (See under Reaction in Chapter on Media.) Yeasts, moulds and a few bacteria (*e. g.*, pertussis bacillus, p. 122; tubercle bacillus, p. 122) grow best on a slightly acid medium. An amount of acid or alkali insufficient to prevent the development of bacteria may still suffice to rob them of some of their most important functions, such as the production of enzymes. The different effect upon closely allied varieties of bacteria of a slight excess of acid or alkali is sometimes made use of in separating those which may be closely allied in many other respects.

[1] Rettger, Berman and Sturges: Jour. Bact., 1916, **2**, 15; Newer Knowledge, Chicago Univ. Press, 1928 (also see other chapters in this volume on Metabolism of Bacteria).
[2] Koser and Rettger: Jour. Inf. Dis., 1919, **4**, 301.
[3] Physiol. Rev., 1923, **3**, 438.
[4] Thyotta and Avery: Proc. Soc. Exp. Biol. and Med., 1920–1921, p. 197.

Action of One Species Upon Another.—In nature, microörganisms usually occur in mixed cultures (*e. g.*, in water, milk, intestinal contents of all animals), and here we may see antagonistic action in the prevalence of one species over others (*e. g.*, the lactic acid formers in the intestines), or coöperative action in the equal and luxuriant growth of two or more species (*e. g.*, pneumococcus and influenza bacillus in the lungs). The effects of so-called symbiosis among microörganisms have been emphasized by several investigators, particularly by Castellani.[1]

Experimentally, the existence of antagonisms can be demonstrated by inoculating alternate streak cultures of various bacteria on gelatin or agar media. It is found that many species will grow not at all or only sparingly when in close proximity to some other species. This antagonism, however, is often only one-sided in character and may be due to such definite causes as the production of acid from the fermentation of sugars. Again, when gelatin or agar plates are planted with a mixture of two species of bacteria, it may be observed that only one of the two grows. A third method of making this experiment is simultaneously to inoculate the same liquid medium with two species, and then to examine them later, both microscopically and by making plate cultures; not infrequently one species may take precedence over the other which after a time it may entirely overcome.

The *stimulating* (symbiotic) or *coöperative* (commensal) action of microörganisms may be demonstrated experimentally in the following examples:

(*a*) Pneumococci, when grown together with a bacillus obtained from the throat, produce very large, succulent colonies. The influenza bacillus, which will not grow alone upon ordinary nutrient agar, will grow well there in the presence of certain other bacteria. Some anaërobic species grow even with the admission of air if only some aërobic species are present (tetanus bacilli with diphtheria bacilli).

(*b*) Certain chemical effects, as, for instance, the decomposition of nitrates, cannot be produced by many species of bacteria alone, but only when two are associated.

Behavior toward Free Oxygen and Other Gases.—The majority of microörganisms require varying amounts of free oxygen for their growth, but a considerable minority fail to grow unless it is included. This latter fact, noted first by Pasteur, led him to divide germs into aërobic and anaërobic forms. Between these two groups we have those that can grow both with and without free oxygen. Organisms that can grow under conditions other than the most favorable are called facultative organisms.

(*a*) **Aërobic Organisms.** — Growth is most abundant only in the presence of free oxygen. The slightest restriction of air lessens development. The anthrax bacillus, the hay bacillus and many common air bacilli are examples of this class. Spore formation, especially, requires the free admission of air.

[1] Fungi and Fungus Diseases, Am. Med. Assoc., Chicago, 1928, p. 81.

(*b*) **Anaërobic Organisms.**—Growth and spore formation only on the practical exclusion of free oxygen. Among this class of organisms are the bacillus of malignant edema, the tetanus bacillus, the bacillus of symptomatic anthrax, and many soil bacteria. Exposed to the action of oxygen, the vegetative forms of these bacteria are readily destroyed; the spores, on the contrary, are very resistant. Anaërobic germs being deprived of free oxygen—the chief source of energy used by the aërobic species to oxidize the nutritive substance in the culture media—are dependent for their oxygen upon decomposable substances, such as grape-sugar.

(*c*) **Facultative Anaërobic and Facultative Aërobic Organisms.**—The greater number of aërobic germs, including most of the pathogenic species, are capable of withstanding, without being seriously affected, some restriction in the amount of free oxygen admitted (facultative anaërobes), and some grow equally luxuriantly under both conditions. Life in the animal body, for example, in the intestines, necessitates existence with diminished supply of oxygen. If in any given variety of bacteria the amount of oxygen present is unfavorable, there will be more or less restriction in some of the life processes of this variety, such as pigment and toxin production, spore formation, etc. Pigment formation almost always ceases with the exclusion of free oxygen and proteolysis of facultative anaërobes is lessened.

It has been observed not infrequently that certain species which on their isolation at first show more or less anaërobic development—for instance, a preference to grow in the depth of a shake agar-tube culture—after a while seem to become markedly aërobic, growing abundantly on the surface of the medium (facultative aërobes).

Micro-aërophilic Organisms.—Those organisms that grow best or grow exclusively when the oxygen is only partly removed, *e. g.*, at a certain layer in a tube of deep semisolid medium, are called *micro-aërophilic organisms.*[1]

Other Gases.—While all facultative organisms as well as strict anaërobes grow well in nitrogen and hydrogen, they behave very differently toward carbonic acid gas. A large number of these species do not grow at all, being completely inhibited in their development until oxygen is again admitted—for example, B. anthracis and B. subtilis and other allied species. It has been found in some species, as glanders and cholera, that the majority of the organisms are quickly killed by CO_2, while a few, such as staphylococci, offer a great resistance, rendering impossible complete sterilization by means of this gas. The streptococcus as well as the staphylococcus exhibits a scanty growth. A mixture of one-fourth air to three-fourths carbonic acid gas seems to have no injurious effect on bacteria which cannot grow in an atmosphere of pure CO_2. Under pressure CO_2 is more effective (page 61).

Sulphuretted hydrogen in large quantity is a strong bacterial poison. Even in small amounts it kills some bacteria.

[1] Lyon, in Science, 1917, **65**, 19, suggests that the word oligaërobic is better than the word micro-aërophilic.

Effect of Temperature.—Some form of microbic life is possible within the limits of 0° and 70° C. The maximum and minimum temperature for each individual species ordinarily lies from 10° to 30° C. apart, and the optimum covers about 5° C. Usually the temperature of the soil in which the germs are deposited is the controlling factor in deciding whether growth will or will not take place. Thus, nearly all parasitic microörganisms require for development a temperature near that of the body of their host, while most saprophytic forms grow best at temperatures lower than 37° C. Microbes when exposed to lower temperature than suffices for their growth, while having their activities decreased, may not be otherwise injured unless actually frozen for a certain time; when exposed to higher temperatures than allows of growth the life of the organism is more or less quickly destroyed. Sudden marked changes in temperature are detrimental.

Microörganisms have been classified according to the temperatures at which they develop, as follows:

Psychrophilic Microbes.—Minimum at 0° C., optimum at 15° to 20° C., maximum at about 30° C. To this class belong many of the water microörganisms, such as the phosphorescent bacteria in sea-water; a number of saprogenic microbes and many moulds and yeasts.

Mesophilic Microbes.—Minimum at 5° to 25° C., optimum about 37° C., maximum at about 43° C. To this class belong all pathogenic microbes of warm blooded animals, most parasitic and a number of saprogenic forms.

Thermophilic Microbes.—Minimum at 25° to 45° C., optimum at 50° to 55° C., maximum at 60° to 70° C. This class includes a number of soil bacteria which are almost exclusively spore-bearing bacilli. They are also found widely distributed in feces.

By carefully elevating or reducing the temperature the limits within which a species will grow can be altered. Thus, the anthrax bacillus may be gradually made to accommodate itself to a temperature of 42° C., and pigeons, which are comparatively immune to anthrax, partly on account of their high body temperature (42° C.), when inoculated with this anthrax succumb to the infection. Another culture accustomed to a temperature of 12° C. kills frogs kept at 12° C. We have cultivated a very virulent diphtheria bacillus so that it will grow at 43° C. and produce strong toxin.

Effect of Low Temperature.—Temperatures even far under 0° C. are only slowly injurious to microörganisms, different species being affected with varying rapidity. This has been demonstrated by numerous experiments in which they have been exposed for weeks in a refrigerating mixture at —18° C. If a culture of typhoid bacilli is frozen, about 50 to 70 per cent. of the organisms are killed at the time. At the end of one week not more than 10 per cent. survive, and at four weeks not over 1 per cent. After six months none survives. More resistant bacteria live longer and spores may survive in ice for years. Bacteria have even been subjected to a temperature of —175° C. by immersing them in liquid air kept in an open tube for two hours, and

15 to 80 per cent. were found still to grow when placed in favorable conditions. We found about 10 per cent. of typhoid bacilli alive after thirty minutes' exposure to this low temperature. Staphylococci were more resistant. Spores were scarcely affected at all.[1]

Effect of High Temperatures (See Table on Resistance of Microörganisms, Section IV).—Prolonged temperatures from 5° to 10° C. over the optimum affect microörganisms injuriously in several respects. For instance, varieties may be produced of diminished activity of growth, the virulence or the property of causing fermentation may be decreased, and the power of spore formation may be gradually lost.

If the maximum temperature is exceeded, the organism dies. The thermal death-point for the psychrophilic species is about 37° C., for the mesophilic species about 45° to 55° C., and for the thermophilic species about 75° C. There are no non-spore-bearing bacteria, except possibly a few cocci, which when moist are able to withstand a temperature of 100° C. even for a few minutes. A long exposure to temperatures between 60° and 80° C. has the same result as a shorter one at the higher temperatures. Ten to thirty minutes' exposure to moist heat will at 60° C. kill the cholera spirillum, the streptococcus (see Pasteurization of Milk), the typhoid bacillus and the gonococcus and at 70° C. the staphylococcus, the latter being among the most resistant of the pathogenic organisms which show no spores. A much shorter exposure will kill a large percentage of any mass of these bacteria.

Effect of Dry Heat.—When microörganisms in a desiccated condition are exposed to the action of heated dry air, the temperature required for their destruction is much above that required when they are in a moist condition or when they are exposed to the action of hot water or steam. A large number of pathogenic and non-pathogenic species are able occasionally to resist a temperature of over 100° C. dry heat for from ten minutes to one hour. In any large number of bacteria a few are always more resistant than the majority. A temperature of 120° to 130° C. dry heat maintained for one and a half hours will destroy all bacteria in the absence of spores. Bacteria freed from surface moisture and preserved in oil are very resistant to heat.

Resistance of Spores to Heat.—Spores possess a great power of resistance to both moist and dry heat. Dry heat is comparatively well borne, many bacterial spores resisting a temperature of over 130° C. for as long as three hours. Exposed to 150° C. for one hour, practically all spores are killed. Moist heat at a temperature of 100° C., either boiling water or free-flowing steam, destroys the spores of most varieties of bacteria within fifteen minutes; certain pathogenic and non-pathogenic species, however, resist this temperature for hours. The spores of a bacillus from the soil were destroyed after five and a half or six hours' exposure to streaming steam. They were destroyed, however, by exposure for twenty-five minutes in steam at 113° to 116° C. and in two minutes at 127° C. The spores from tetanus bacilli may require twenty minutes' exposure to kill them. Spores in fatty

[1] See Hilliard and Davis, Jour. Bacteriol., 1918, **3**, 423, for bibliography.

media are more resistant to heat, therefore immersed in oil they may be killed with great difficulty.

The resistance of spores to moist heat is tested by suspending threads, upon which the spores have been dried, in boiling water or steam. The threads are removed from minute to minute and laid upon agar or in broth and kept at a suitable temperature for the germination of any living spores. (See chapter on Disinfection.)

Influence of Chemicals.—The deleterious effect of chemicals, especially those used as germicides, will be considered under Disinfection. The influence of dyes, researches on which have been so stimulated by the studies of Churchman,[1] is taken up in Chapter on Media.

Tactic Effect of Chemicals.—*Chemotaxis.*—Some substances exert a peculiar attraction for microörganisms, known as *positive chemotaxis,* while others repel them—*negative chemotaxis.* Morevoer, all varieties are not affected alike. Oxygen, for example, attracts aërobic and repels anaërobic bacteria, and for each variety there is a definite proportion of oxygen which most strongly attracts. The chemotactic properties of substances are tested by pushing the open end of a fine capillary tube, filled with the substance to be tested, into the edge of a drop of fluid containing the organisms and examining under the microscope. We are able thus to watch the action of the microbes and note whether they crowd about the tube opening or are repelled from it. Among the many substances showing positive chemotaxis for nearly all microorganisms are peptone and urea, while among those showing negative chemotaxis are alcohol and many of the metallic salts. Such experiments are, of course, rough. The diffusion of the substances from the tube into the surrounding medium must play an extremely active rôle in the final result.

Influence of Light.—A large number of microörganisms are inhibited in growth by the action of bright daylight, more are affected by direct sunlight, and when the action of the sun's rays is prolonged they lose their power of developing when later placed in the dark.

Some motile organisms move toward the point of greatest luminosity, others away from it. The violet and blue rays are more active than other parts of the spectrum in determining motion.

The susceptibility of bacteria to light may be tested by suspending a large number of bacteria in nutrient gelatin or agar and pouring the media while still fluid into Petri dishes, upon each of which has been pasted a strip of black paper on the side exposed to the light. The action of heat may be excluded by allowing the rays of light to pass through a layer of water or alum of several centimeters' thickness. After the plates have been exposed to the light for one-half, one, one and a half, two hours, etc., they are taken into a dark room and allowed to stand at 20° to 35° C. a sufficient length of time to allow for growth, and then examined to see whether there are colonies anywhere except on the parts covered by the paper; when the colonies exposed to the

[1] Churchman: Jour. Exp. Med., 1912, **16,** 221 and 822; 1913, **17,** 373 and **18,** 187; 1921, **33,** 569.

light have been completely destroyed, there is lying in a clear sterile field a sharply defined region of the shape of the paper strip crowded with colonies.

Protected by ordinary non-colored glass the sun's rays act very slowly.

Only the ultraviolet, violet and blue rays of the spectrum possess bactericidal action; green light has very much less power; red and yellow light, none at all. The action of light is apparently assisted by the admission of air; anaërobic species, like the tetanus bacillus, and facultative anaërobic species, such as the colon bacillus, are able to withstand quite well the action of intense, direct sunlight in the absence of oxygen, for four hours.

According to Richardson and Dieudonné, the mechanism of the action of light may be at least partially explained by the fact that in agar plates exposed to light for a short time (even after ten minutes' exposure to direct sunlight) hydrogen peroxide (H_2O_2) is formed. This is demonstrated by exposing an agar plate half-covered with black paper, upon which a weak solution of iodide of starch is poured, and over this again a dilute solution of sulphate of iron; the side exposed to the light turns blue black. In gases containing no oxygen, hydrogen peroxide is not produced, and the light has no injurious effect. Access of oxygen also explains the effect which light produces on culture media which have been exposed to the action of sunlight, as standing in the sun for a time, when afterward used for inoculation. Some bacteria subsequently introduced into such media grow badly —far worse than in fresh culture media which are kept in the shade.

Influence of Radium.—*Radio-active fluids* have a slight inhibiting effect on microbal growth, but nothing decided enough to be used for therapeutic purposes has been evolved up to the present time.

Influence of X-rays.—These rays have a slight inhibiting effect on microörganisms when they are directly exposed to them. The effect of the rays on tissues, however, may influence indirectly the course of an infection. Bowditch's[1] work on pertussis awaits corroboration.

Influence of Electricity.—The majority of the observations heretofore made on this subject would seem to indicate that there is no direct action of the galvanic current on bacteria; but the effect of heat and the electrolytic changes in the culture liquid resulting from the electrolysis may destroy them.

Protozoa may be contracted by moderate induction shocks and killed by strong ones.

The effect of hydrogen-ion concentrations on the rate and direction of flow of microörganisms in an electric current has been studied by Northrup,[2] Falk[3] and others.

When the current of electricity is passed through a liquid neutral medium most active protozoa swim with their long diameters in the

[1] Bowditch and Leonard: Boston Med. and Surg. Jour., 1923, **188**, 312.
[2] Jour. Gen. Physiol., 1922, **4**, 629.
[3] Newer Knowledge, Chicago Univ. Press, 1928.

direction of the lines of force to assemble behind the cathode. Some flagellates and a few ciliates, however, move toward the anode. The direction of motion has been shown by Dale to vary with the nature and concentration of the medium. Most bacteria move toward the anode. Vaccine virus collects about the cathode.

Influence of Agitation.—Meltzer has shown that the vitality of bacteria is destroyed by protracted and violent shaking, which causes a disintegration of the cells. Many species are more quickly autolyzed after violent shaking. Hence shaking, which was earlier thought to be helpful in the production of bacterial vaccines, is no longer used.

Influence of Pressure.—Microörganisms in fluids which are subjected to great pressure are for a time inhibited in their growth. Larson found that a direct pressure of 6000 atmospheres kills non-spore-bearing bacteria in fourteen hours, and that a pressure of about 12,000 atmospheres for the same length of time is required to kill spore-bearing bacteria.[1]

Osmosis.—Osmosis, due to differences of pressure between the medium and the microörganisms and to the permeability of the cell membrane for different substances, is a constant and essential phenomenon of life. Presumably the normal development of an organism takes place when the osmotic pressure within the cell is equal to (isotonic) that of its medium. When an organism is transferred to a new medium with an osmotic pressure markedly different from that of the old one, decided changes in morphology may occur. If the difference is too great or the transfer is too sudden, death may result. If the new medium has a higher pressure, then water is abstracted from the cell and the protoplasm shrinks from its membrane. This is called *plasmolysis*. When the new medium has a lower pressure than the old the cell may burst. This process is called *plasmoptysis*.

Influence of Carbonic Acid under Pressure.—Some non-spore-bearing bacteria are killed by CO_2 of 50 atmospheres pressure in about one and a half hours. Others are more resistant. D'Arsonval and Charrin submitted a culture of B. pyocyaneus to a pressure of 50 atmospheres under carbonic acid. At the end of four hours cultures could still be obtained, but the bacillus had lost its power of pigment production. A few colonies were developed after six hours' exposure to this pressure, but after twenty-four hours no development occurred. We have subjected broth and milk containing typhoid, dysentery, diphtheria and colon bacilli to the gas under a pressure of 75 and 150 pounds. Within twenty-four hours 99 per cent. of those in the broth and 98 per cent. of those in the milk were destroyed. Within one week the broth was sterile and within four weeks the milk was sterile. Tubercle bacilli and staphylococci were much more resistant, but little effect was noticed in twenty-four hours. The results were the same whether the cultures were kept at 10° or 25° C. Yeast cells withstand the action of CO_2 for more than twenty-four hours.[2] Bottled waters charged with carbonic acid are usually sterile.

[1] Larson, Hartzell and Diehl: Jour. Infect. Dis., 1918, **22**, 271. [2] Ibid.

Effect of Drying.—For growth, microörganisms require much moisture. Want of water affects them in different ways. Upon dried culture media development soon ceases but if, after growth has occurred, the culture media and organisms are dried quickly, the organisms remain viable for a much longer time than on media dried gradually. The question as to how long the non-spore-bearing forms are capable of retaining their vitality when dried on a cover-glass or silk threads has been variously answered. We know now that there are many factors which influence the retention of vitality. Spores, of course, are more resistant than vegetative forms.

The varying results sometimes reported by different observers may be explained by the fact that the conditions under which they were made were different, depending upon the desiccator used, the medium upon which the cultures were grown, and the use of silk threads or cover-glasses. In all these experiments, of course, it should be previously determined that in spore-bearing species there are no spores present. Even when a dried culture lives for a long time the majority of the organisms die in a few hours after drying. We have found 1,500,000 colon bacilli to be reduced to 100,000 after three hours' drying. In tissues or exudates they resist drying much longer than when unprotected.

Encysted protozoa withstand long periods of desiccation. Most forms when dried quickly remain viable much longer than when dried slowly.

Duration of Life in Pure Water.—When microörganisms which require much organic food for their development (and these include most of the pathogenic species) are placed in distilled water, they soon die—that is, within a few days. Their death is largely due to plasmoptysis. Even in sterilized well-water or surface water their life duration does not usually exceed eight to fourteen days, and they rarely multiply. Instances, however, of much more extended life under certain conditions are recorded.

RESULTS OF MICROBAL GROWTH.

Microörganisms are not only influenced by their surroundings, but in their growth activities they produce changes that affect not only themselves but also their environment. Among the innumerable species of germs in Nature, only a small percentage of them are harmful to man. Large groups perform activities that are essential to his life, while other groups aid him, both directly and indirectly in various ways. Thus, a number of species of microbes break up the dead organic matter of animals and plants into simpler products capable of being taken up again as food by plants and so by animals. Another group—certain plant-root parasites—help to form simple nitrogen compounds by the assimilation of atmospheric nitrogen. (See below).

In all of these processes *heat* is formed. It does not, however, attract attention in our usual cultures because of its slight amount. Even fermenting culture liquids with abundance of bacteria cause no sensation of warmth when touched by the hand. Careful tests, however, show that heat is produced. The increase of temperature in

organic substances when stored in a moist condition, as tobacco, hay, manure, etc., is due, partly at least, to the action of bacteria.

Production of Light.—Microörganisms which have the property of emitting light (*photogens*) are quite widely distributed in nature, particularly in media rich in salt, as in sea-water. Many of these have been accurately studied. The emission of light is a property of the living protoplasm of the organism, and is not usually due to the oxidation of any photogenic substance given off by them; at least only in two instances has such substance been claimed to have been isolated. While these organisms cannot emit light except during life, they can live without emitting light. They are best grown under free access of oxygen in a culture medium prepared by boiling fish in sea-water (or water containing 3 per cent. sea-salt), to which 1 per cent. peptone, 1 per cent. glycerin, and 0.5 per cent. asparagin are added. The power of emitting light is soon lost unless the organism is frequently transplanted to fresh media.

Chemical Changes.—It is still difficult to classify with clearness the chemical changes caused by microörganisms, because of our lack of definite knowledge of all their processes of metabolism. Thus, as a result of the growth of a microbe in Nature, both destructive and constructive changes are continually going on in its surroundings depending in character upon the kind of microbe and surroundings; and these in turn may cause destructive changes in this microbe and constructive changes in other microbes and so on. These processes may be broadly designated as oxidations, reductions, condensations and polymerizations. In all of these changes active hydrogen and the hydroxyl ions play an important, if not the chief part.[1] Probably most if not all of these changes are initiated and speeded by intracellular and extracellular substances, such as enzymes produced by the organisms. Some such tabulation as the following might be of aid.

A. Changes produced by appropriating substances of a simple chemical composition directly from the environment.
   1. Oxygen from,
      (*a*) Nitrates, producing nitrites then N, a reduction process called denitrification.
      (*b*) Sulphates, producing sulphites then S, also a reduction process.
      (*c*) Air and water helping in both oxidizing and reducing processes.
      (*d*) From the splitting of other compounds containing oxygen.
   2. Nitrogen from air directly or through root nodules producing nitrites and nitrates, called nitrification.
   3. NH_3 and CO_2 from decomposing organic matters.
   4. H_2S by oxidation depositing sulphur within cell (sulphur bacteria).

[1] Dakin, H. D.: Oxidations and Reductions, 1922. Beatty, J.: The Method of Enzyme Action, Philadelphia, 1917. Falk: The Chemistry of Enzyme Action, 1921, also Falk and Bergeim: Practical Physiological Chemistry, 9th ed., P. Blakiston's Sons, 1927. Waksman and Davison: Enzymes, Williams & Wilkins Company, 1926.

B. By the building up of certain complex organic products of cell activity, none of which have been isolated pure.

1. Non-specific "endoproteins."
2. Specific endoproteins (endotoxins, agglutinogens, etc.).
3. Substances secreted and perhaps excreted that may or may not be essential to life of cell.

(*a*) *Enzymes* (Biological catalysts, ferments). Initiate and accelerate chemical reactions, under favorable conditions, either in cell or in medium without themselves being (permanently) chemically changed. They may be freely diffused from the cell (exoenzymes) or they may be bound up in the cell and only obtained by rupture of the cell (endoenzymes). Antienzymes have been demonstrated. The following are some of the more common microbal enzymes. They are usually given the name of the substance split or of the action produced plus the suffix *ase*.

(1) *Proteases* (proteoclastic or proteolytic enzymes) producing a hydrolytic cleavage of protein compounds with the formation of various split products, *e. g.*, indol, skatol, phenol, volatile fatty acids, proteoses, peptones, peptids, amino-acids and finally NH_3, H_2S, CO_2H, H_2O_2 (if sufficient oxygen and little catalase), H_2O, O; under anaërobic conditions, more aromatic and malodorous alkaline products are formed, particularly mercaptans, and ptomaines. This has been called *putrefaction*. Under aërobiosis few foul-smelling substances are formed. This has been called *decay*.

Gelatinase causes liquefaction of gelatines but not digestion of egg albumen or fibrin.

(2) *Coagulase* (rennin-like or "lab" enzyme) producing coagulation of milk independent of the presence of acids. It splits casein into paracasein.

(3) *Carbohydrases,* Carbohydrate-splitting enzymes (fermentation in a narrow sense); (*a*) amylases split starch and dextrin to maltose; (*b*) inulases split inulin to fructose; (*c*) lactases split lactose to glucose and galactose; (*d*) maltases split maltose to glucose; (*e*) sucrases split sucrose to glucose and fructose; (*f*) zymases split sugars to ethyl alcohol, CO_2, etc.

(4) *Lipases* (fat-splitting enzymes) split fats to fatty acids and glycerol. The rancidity of butter may occasionally be due to a bacterial lipase.

(5) *Oxidases and Peroxidases* assist oxidations. Catalase which breaks up H_2O_2 with liberations of oxygen is put with these.

(6) *Hydrogenases* assist reductions.

(7) *Carboxylases,* split COOH groups of aliphatic acids to CO_2; as in the formation from amino-acids of amines.

(*b*) Substances having some of the properties of enzymes:

(1) Lytic substances (*a*) hemolysins; (*b*) other cytolysins including the autolysin of Twort and d'Herelle ("Bacteriophage").

(2) Specific exotoxins stimulating the production of antitoxin in animals.

(3) Vitamins.

(*c*) Non-specific toxic substances (x substances of Zinsser, Parker and Kuttner).

C. By the production and storing of substances not directly necessary for the life of the cell.

1. Pigments (*a*) which diffuse into medium or (*b*) which are not soluble in the medium used and therefore condense about bodies of microbes.
2. Iron stored through oxidizing processes by special bacteria (iron bacteria).
3. Fats as in B. tuberculosis.

Fermentation.—Fermentation may be defined broadly as a chemical decomposition of an organic compound, induced or hastened by chemical substances called ferments or enzymes which are elaborated by the organisms. The reactions may be summed up as hydrolytic and the opposite—oxidative—causing rearrangement of the H and OH radicals (see Beatty). Fermentation demands the proper nutriment, temperature, moisture, and the absence of deleterious substances. The enzyme itself is not markedly diminished in quantity while fermentation is taking place, though the process yields products that inhibit its action; hence fermentation ceases when these products are in excess, or when the nutriment is exhausted. That the process will often begin again after diluting the fermented medium, shows that the *concentration* of the harmful products plays an important part in the inhibitory action.

Characteristics of Enzymes.—Enzymes are non-dialyzable (colloidal) products of living protoplasm that are recognized only by their activities. They are soluble in water and in glycerine. They may be precipitated with other substances from their solutions either by alcohol or by certain salts, such as ammonium or zinc sulphate. They are easily carried down by different precipitates. They withstand moderate dry heat, but are usually destroyed in watery solutions on exposure of ten to thirty minutes to a temperature of 60° to 70° C. Upon melting after

freezing their activity is not impaired. They are sensitive to acids, and are slightly less so to alkalies. Strong acids or alkalies destroy them. They are able, even when present in minute quantities, partly to split up or decompose complex organic compounds into simpler substances, and thus to render the foodstuff suitable for plant growth.

As an example of enzyme action we may take any of the *sugar-splitting enzymes* (*zymase, lactase, maltase, etc.*). Many bacteria and yeasts are capable of splitting sugars, especially under anaërobic conditions. The products vary with the organisms and other conditions. The reaction may be indicated by the following equations:

$$\underset{\text{Grape-sugar.}}{C_6H_{12}O_6} \rightarrow \underset{\text{2 Alcohol.}}{2C_2H_6O} + \underset{\text{2 Carbon dioxide.}}{2CO_2}$$

Or,

$$\underset{\text{Grape-sugar.}}{C_6H_{12}O_6} \rightarrow \underset{\text{2 Lactic acid.}}{2C_3H_6O_3} \rightarrow \underset{\text{3 Acetic acid.}}{3C_2H_4O_2}$$

Or,

The action of ferments is largely specific but not entirely. It seems to be a group specificity. While the action with sufficient water in the substrate is hydrolytic, with a limitation of water synthetic processes are carried out. The essential character of enzyme action is catalytic. The differences between enzymes and the inorganic catalysts are probably largely due to the complex system in which they act.

The velocity of enzyme action depends in general upon the concentration of the enzyme and of the menstruum, and the amount of electrolytes present.

Formation of Acids and the Aldehyde Group from Carbohydrates.—Free acids or hydrogen ions and the aldehyde group are formed by many microbes in culture media containing some form of sugar or other fermentable carbohydrates, such as the alcohol mannite.

Among the acids produced, the most important is lactic acid; also traces of formic acid, acetic acid, propionic acid and butyric acid, and not infrequently some ethyl alcohol and aldehyde or acetone are formed. Occasionally no lactic acid is present.

Formation of Gas from Carbohydrates and other Fermentable Substances of the Fatty Series.—The only gas produced in *visible* quantity in sugar-free culture media is nitrogen. If sugar is vigorously decomposed by bacteria, as long as pure lactic acid or acetic acid is produced there may be no development of gas, as, for instance, with the B. typhosus on grape-sugar, but frequently there is much gas developed, especially in the absence of air. About one-third of the acid-producing species also develop gas abundantly, this consisting chiefly of CO_2, which is always mixed with H. Marsh-gas is seldom formed by bacteria, with the exception of those decomposing cellulose. (For demonstration see Chapter IV.)

Formation of Acids from Alcohol and other Organic Acids.—It has long been known that B. aceti and allied bacteria convert dilute solutions of ethyl alcohol into acetic acid by oxidization:

$$\begin{array}{l} CH_3 \\ | \\ CH_2OH \end{array} + O_2 = \begin{array}{l} CH_3 \\ | \\ COOH \end{array} + H_2O$$

The higher alcohols—glycerol, dulcitol, mannitol, etc.—are also converted into acids.

Finally, numerous results have been obtained from the conversion of the fatty acids and their salts into other fatty acids by bacteria.

Pigment Production.—Pigments have no known importance in connection with disease, but are of interest and have value in identifying bacteria. Very little is known of their chemical composition. They are usually stored up around the bacterial cells, but some are diffused throughout the medium. They are of almost every hue. A few only are given here.

Red and Yellow Pigments.—Of the twenty-seven red and yellow chromogenic bacteria studied by Schneider, almost all produce pigments soluble in alcohol and insoluble in water. The large majority of these pigments possess in common the property of being colored blue-green by sulphuric acid and red or orange by a solution of potash. Though varying considerably in their chemical composition and in their spectra, they may be classified, for the most part, among that large group of pigments common to both the animal and vegetable kingdoms known as *lipochromes*, and to which belong the pigments of fat, yolk of eggs, the carotin of carrots, turnips, etc.

Violet Pigments.—Certain bacteria produce violet pigments, also insoluble in water and soluble in alcohol, but insoluble in ether, benzol and chloroform. These are colored yellow when treated in a dry state with sulphuric acid and emerald green with potash solution.

Blue Pigments.—Blue pigments, such as the blue pyocyanin produced by B. pyocyaneus, and the fluorescent pigment common to many so-called fluorescent bacteria (bacterio-fluorescence) are examples. In cultures the pigment is at first blue; later, as the cultures become alkaline, it is green.

Numerous investigations have been made to determine the cause of the variation in the chromogenic function of bacteria. All conditions which are unfavorable to the growth of the bacteria decrease the production of pigment, as cultivation in unsuitable media or at too low or too high a temperature, etc. The B. prodigiosus seldom makes its red pigment at 37° C., and when transplanted at this temperature, even into favorable media, the power of pigment production is gradually lost. B. pyocyaneus does not produce its blue pigment under anaërobic conditions. Occasionally colored and uncolored colonies of the same species of bacteria may be seen to occur side by side in one plate culture, as, for example, in the case of Staphylococcus pyogenes.

Reduction Processes.—The following processes depend wholly or in part upon the reducing action of nascent hydrogen:

1. *Sulphuretted Hydrogen* (H_2S).—All bacteria, according to Petri and Maassen, possess the power of forming sulphuretted hydrogen, particularly in liquid culture media containing much peptone (5 to 10 per cent.); only a few bacteria form H_2S in bouillon in the absence of peptone, while about 50 per cent. in media containing 1 per cent.

of peptone possess the property of converting sulphur into sulphuretted hydrogen, for which purpose is required the presence of nascent hydrogen. (For demonstration see Chapter IV.)

2. *The Reduction of Blue Litmus Pigments, Methylene Blue and Indigo to Colorless Substances.*—The superficial layer of cultures in contact with the air shows often no reduction, only the deeper layers being affected.

3. *The Reduction of Nitrates to Nitrites, Ammonia and Free Nitrogen.*—The first of these properties seems to pertain to a great many bacteria.

TRANSMISSIBLE LYSIS OF BACTERIA.

(Bacteriophage)

That bacteria and other cells may be dissolved by substances apparently the products of the cells themselves, has been known since the use of bacterial cultures, though only recently has the lytic powers of tears and other tissues been studied.[1] That bacteria may produce in the test-tube solvents for red blood cells and for other bacteria has also been long known, but that bacteria may develop an autolytic substance, indefinitely transmissible, was not brought to our attention until the work of Twort[2] in 1915. Twort[3] while searching for filtrable viruses observed in agar cultures made with glycerinated calf virus certain watery areas. Some of the colonies about these areas could not be subcultured and if left for some time became themselves transparent. If a small portion of such a glassy colony was touched to a pure culture of certain organisms a spreading transparent area started. A filtrate of this transparent material prevented growth when added to a young culture of the original organism. Twort determined other properties of this material and came to the conclusion that it was probably an enzyme derived from the bacteria themselves, but that it might be a filtrable virus, dependent upon the bacteria for growth as amœbæ are under certain conditions. No attention was paid to this work until 1917, when d'Herelle announced as a new phenomenon one that is apparently identical with that of Twort. D'Herelle believed then and he still believes[4] that the lytic principle is a living ultramicroscopic organism (Bacteriophagum intestinale) and gives a list of evidence in favor of this hypothesis.

D'Herelle obtained his first lytic substance from a dysentery stool. A trace of the diluted filtrate from such a stool introduced into a young broth culture of Shiga bacillus, caused a clearing of cloudy culture within a few hours, and transplants remained sterile. A trace of this dissolved culture placed in another young broth culture caused clearing again, and so on through indefinite generations. This technic is given

[1] Flemming: Proc. Roy. Soc., London, 1922, **93**, 606. [2] Ibid., 1915, vol. **11**.
[3] Twort, D'Herelle, Bordet and Others: Brit. Med., Jour., 1922, **11**, 289; contains an instructive symposium on this subject.
[4] Harvey Lecture, 1928.

in detail in his book "Les Bacteriophages."[1] The lytic principle seemed to be more or less specific, acting only on a single species or a few closely related forms.

A number of other workers quickly joined the group of investigators of this phenomenon and now a host of workers are busy at it. The majority agree in thinking it a product of the bacteria of the nature of an enzyme.

Bordet and Ciuca[2] obtained a similar lytic substance by injecting B. coli into the peritoneal cavity of guinea-pigs several times. About forty-eight hours after the last injection the peritoneal fluid, rich in leukocytes, was withdrawn. The cultures gave a number of colonies showing translucent areas which contained the lytic principle. They believe that the lytic substance is developed by the microbe as the result of a reaction between the invading organism and the tissue cells of the host.

Bordet and Ciuca prepared an autolytic serum,[3] which is being studied.

The chief action of the lytic substance on a susceptible culture is to divide the culture into a resistant and a sensitive (lytic carrying) strain. The resistant strains are neither lysogenic nor agglutinable. The sensitive bacilli gradually lose the lysogenic property. To the resistant strains is supposed to be due the fatal termination of the disease.

A number of other variants have been described especially for strains of B. coli.[4]

Phages for many different microbes have been obtained. These are noted under the respective microbes.[5]

The latest knowledge of the phages is well summed up by Bronfenbrenner.[6]

[1] Les Bacteriophages, Paris, 1921.
[2] Compt rend. Soc. Biol., 1920, p. 88.
[3] Compt. rend. Soc. Biol., February 5, 1921.
[4] Gratia: Jour. Exp. Med., 1922, **35**, 287.
[5] Klasterman and Small: Jour. Exp. Med., 1928, **47**, 121.
[6] Newer Knowledge, Univ. of Chicago Press, 1928, p. 525.

The following general references for this whole chapter may also be consulted:

Calkins: Protozoölogy, New York and Philadelphia, 1909.
Craig: Manual of Parasitic Protozoa of Man, 1926, Lippincott.
Hegner and Taliaferro: Human Protozoölogy, 1924, Macmillan & Co.
Jennings: Behavior in Lower Organisms, New York, Macmillan & Co., 1906.
Moore: The Pathology of Infectious Diseases of Animals, 4th edition, New York, 1916.

CHAPTER III.

THE MICROSCOPE AND THE MICROSCOPIC EXAMINATION OF MICROORGANISMS.

THE MICROSCOPE.

If lenses were capable of refracting all light equally, and bringing to a focus in one plane all rays proceeding from one plane in the object the microscope would be a comparatively simple instrument. But simple lenses have several serious optical defects.

1. **Spherical Aberration.**—Points in the plane of the object are imaged on the *curved* surface of the spherical lens. This defect may be somewhat diminished by combining convex and concave lenses, and by restricting the size of the field. Objectives corrected in this way are called *aplanatic.*

2. **Chromatic Aberration.**—This defect is due to the fact that the rays of light vary in their refraction according to their wave length (colors), *e. g.*, the red rays have the longest focus and the violet the shortest. This is considerably corrected by combining planoconcave lenses of flint glass with biconvex lenses of crown glass—*achromatic objectives.*

Still more of a correction is made by combining several different kinds of lenses with a lens of fluorite—*apochromatic objectives.*

Monochromatic light may be employed and thus chromatic aberration may be entirely avoided.

3. **Diffraction.**—Less luminous secondary images about the primary image, due to scratches or foreign particles or other defects, may occasionally occur in the very best lenses.

In order to understand fully the principle of the microscope, works on optics should be consulted.[1]

Different Parts of the Microscope (Figs. 9 and 10).—A complete instrument usually has four oculars, or eye-pieces (*A*) which are numbered from 1 to 4, according to the amount of magnification which they yield. Nos. 2 and 4 are most useful for bacteriological work. The objective—the lens (*B*) at the distal end of the barrel—serves to give the main magnification of the object. For stained bacteria the $\frac{1}{12}$ achromatic oil-immersion lens is regularly employed; for photographic purposes the apochromatic lenses are needed, although even here they are not indispensable. A $\frac{1}{16}$ lens may at times be useful, but hardly necessary; a No. 4 ocular and a $\frac{1}{12}$ lens give a magnification of about 1000 diameters (Fig. 11). For unstained bacteria we employ either the $\frac{1}{12}$ immersion or $\frac{1}{6}$ dry lens, according to the purpose for which we study the bacteria; for the examination of colonies when, as a rule, we do not wish to see individual bacteria but only the general appearance of whole groups, we use lenses of much lower magnification (Fig. 12).

[1] Gage: The Microscope, 1925, 14th ed., The Comstock Publishing Company.

The stage (*C*)—the platform upon which the object rests—should be large enough to support the Petri plates if culture work is to be done. The distance between the optical axis of the instrument and the pillar must be great enough to permit one to examine rather more than half the surface of the Petri dish without revolving it. The iris diaphragm (*D*) opens and closes, and, like the iris of the eye, controls the amount of light. Its opening is diminished or increased by moving a small arm underneath the stage. The reflector or mirror (*E*) placed beneath the stage serves to direct the light to the object to be examined. It has two surfaces—one concave and one plane. The concave surface

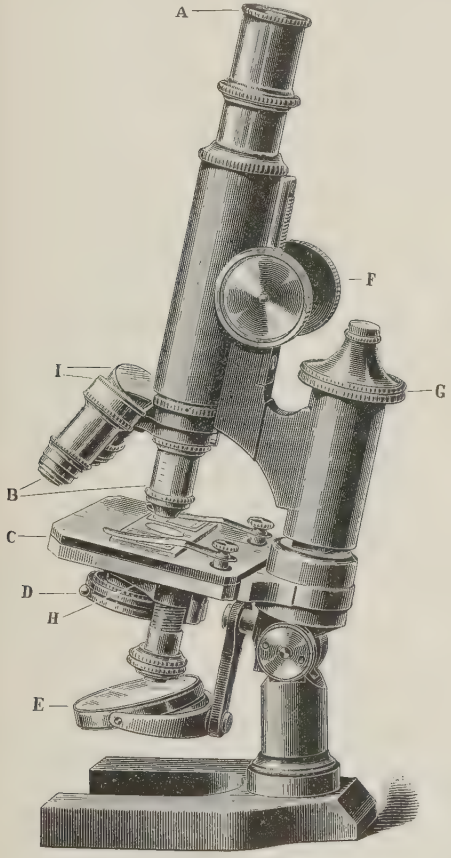


Fig. 9.—Microscope.

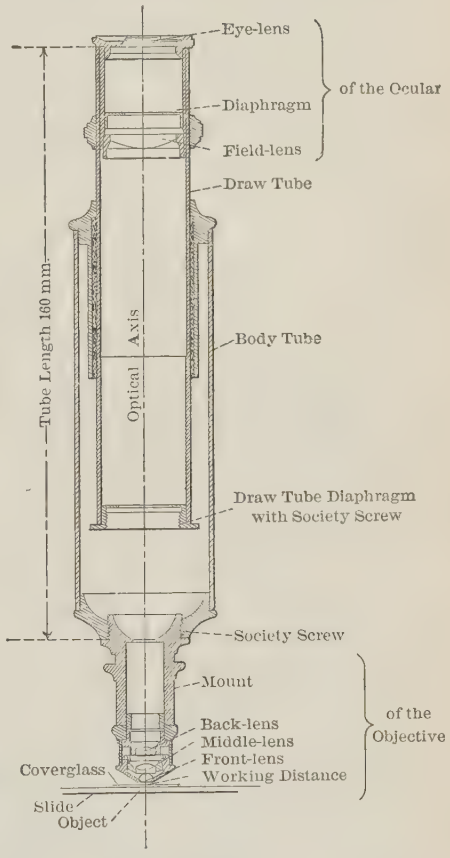


Fig. 10.—Internal structure of the microscope.

must not be employed when the substage condenser is being used, otherwise the rays of light reaching the stage from the condenser will not be correctly focussed. The concave surface may be used when unstained objects, such as colonies or hanging drops are examined. At the same time the Abbé condenser should be lowered and the iris blender (*D*) regulated. The coarse adjustment (*F*) is the rack-and-pinion arrangement by which the barrel of the microscope can be quickly raised or lowered. It is used to bring the bacteria roughly into focus. If the bearings become loose, tighten the little screws at the back of the pinion box. Keep the teeth clean. If the bearings need oiling, use an acid-free lubri-

cant, such as paraffin oil. The fine adjustment (*G*) serves to raise and lower the barrel very slowly and evenly, and is used for the exact study of the bacteria when high-power lenses are used. It is necessarily of limited range and delicate in its mechanism. If, when looking into the eye-piece, no change of focus is noticed by turning the micrometer head, or if the micrometer head ceases to turn, the adjustment has reached its limit. Raise the barrel of the microscope by means of the coarse adjustment, then turn the micrometer back to bring the fine adjustment midway within its range. When the fine adjustment head stops, do not force it. For the microscopic study of microbes it is essential that we magnify the organisms as much as possible and still have their definition clear and sharp. For this purpose the microscope should be provided with an oil-immersion system and a substage condensing apparatus. In using the oil-immersion lens a drop of oil (oil of cedar) of the same index of refraction as the glass is placed upon the face of the lens, to connect it with the cover-glass when the bacteria are in focus. There is thus no loss of light through deflection, as is the case in the dry system. If the lenses become dirty, they should be wiped gently with Japanese lens paper or a clean, soft, old-linen handkerchief. If necessary, breathe on the lens before wiping, and if this does not succeed, use a

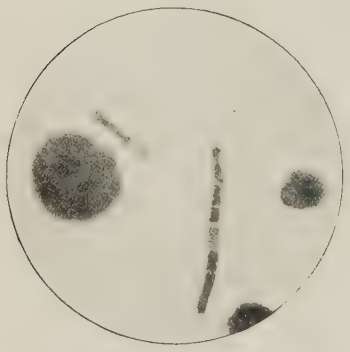


FIG. 11.—Anthrax bacilli and blood cells. × 1000 diameters.

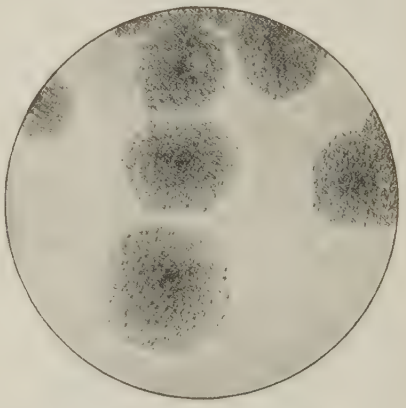


FIG. 12.—Colonies of diphtheria bacilli. × 200 diameters.

little xylol or chloroform. These substances are not to be used unless necessary. An immersion objective should always be cleaned immediately after using. The objective should always be kept covered so as to prevent dust dropping in.

Light.—The best light is obtained from white clouds or a blue sky with a northern exposure. Avoid direct sunlight. If necessary, use white shades to modify the sunlight. Artificial light has one advantage over daylight in that it is constant in quality and quantity. The Welsbach burner and a whitened incandescent bulb give a good light. A blue glass between the artificial light and the lens is often of value. An eye shade may be helpful.

Substage Condensing Apparatus (*H*) is a system of lenses situated beneath the central opening of the stage. It serves to condense the light passing from the reflector to the object in such a way that it is focussed upon the object, thus furnishing the greatest amount of luminosity. Between the condenser and the reflector is placed the iris diaphragm.

Focussing.—This is only difficult with the higher magnifications. Focus the body tube down by means of the coarse adjustment until the objective approaches very near to the cover-glass, being careful not to touch it. Observe from the side where the tip of the objective casts a shadow on the cover-glass. Then with the eye at the eye-piece focus up carefully with the coarse adjustment

until the specimen comes plainly into view. Be careful not to pass this focal point. It is easily unnoticed if the light is too intense and the specimen thin and transparent. If the sliding-tube coarse adjustment is used, focus carefully by giving the tube a spiral movement.

When the object is brought fairly well into focus by means of the coarse adjustment, use the fine adjustment to focus on the particular spot desired, for if this spot is in the center of the field of the low power it should be somewhere in the field of the higher power. It is too much to ask of the maker that the lenses be made absolutely parfocal and centered. The delicacy of the centering can be appreciated when the magnification and the extremely small portion examined are considered. When the objectives are not thus fitted to the nose-piece, refocussing and again hunting up the object are necessary. In so doing we repeat the caution always to focus up before turning the nose-piece. When no revolving nose-piece is used, the change of objectives means the unscrewing of one and the screwing of the other into its place and refocussing.

The beginner should always use the low-power objectives and oculars first. The low-power objectives have longer working distances and are not so apt to be injured. They always show a larger portion of the specimen and thus give one a better idea of the general contour. After obtaining this general idea the higher powers can be used to bring out greater detail in any particular part. Generally speaking, it is best to use a high-power objective and low-power eye-piece in preference to a low-power objective and high-power eye-piece. In the latter case any imperfections in the objective are magnified unduly by the eye-piece, giving, as a rule, poor definition.

Tube Length and Cover-glass.—All objectives are corrected to a certain tube length (160 mm. by most makers—Leitz, 170 mm.) and all objectives in fixed amounts of over 0.7 N. A. are corrected to a definite thickness of cover-glass as well (Zeiss, 0.15 mm., 0.2 mm.; Leitz, 0.17 mm.; Bausch & Lomb and Spencer, 0.18 mm.). These objectives give their best results only when used with the cover-glass and tube length for which they are corrected. As indicated in Fig. 10 the tube length extends from the eye-lens of the eye-piece to the end of the tube into which the objective or nose-piece is screwed. If a nose-piece is used, the draw tube must be correspondingly shortened. If the cover-glass is thinner than that for which the objective is corrected, the tube must be lengthened to obtain best results; if thicker, shortened.

The more expensive objectives are provided with adjustable mounts by which the distances between the lens systems may be changed to compensate for difference of thickness of cover. They are successfully used only in the hands of an expert. One of them out of adjustment is worse than an ordinary objective.

Dark-ground Illumination and the Examination of Ultramicroscopic Particles.—The original apparatus constructed by Siedentopf and Zsigsmondy for the examination of very minute particles (so-called ultramicroscopic, *i. e.*, 1mμ or one millionth of a millimeter) was simplified by Reichert,[1] by devising another condenser. The light which illuminates the object has a greater refraction than the cone of light entering the objective which produces the image.

With this apparatus such living organisms as the *Treponema pallidum* and the flagella on certain bacteria, which can scarcely be seen by ordinary microscopes on account of their low refractive indices, also ultramicroörganisms ultramicroscopic in size, may be demonstrated with great clearness.

Directions for Use.—The condenser is screwed into the place of the Abbé condenser. The preparation is made upon a slide and covered with a coverslip as usual. A drop of oil is then placed upon the upper surface of the condenser, and the slide is laid upon it so that an even layer of oil without air bubbles intervenes between the top of the dark chamber and the bottom of the slide.

The use of *microphotography with ultraviolet light* (according to A. Kohler[2])

[1] Jour. Royal Micro. Soc., 1907, p. 364.
[2] Ztschr. f. wiss. Mikros., 1904, **21**, 129.

makes visible particles that cannot be seen by ordinary light, because of the inability of the violet rays to pass through certain substances, *e. g.*, chromatin.

This method is said to increase 40,000 times our present limit of vision. The few discoveries claimed by these means for diseases of unknown origin have so far lacked sufficient corroboration to constitute them proved.

MICROSCOPIC METHODS.

The direct microscopic examination of suspected substances for microorganisms can be made either with or without staining. Unstained, the microbes are examined living in a hanging drop or on transparent solid media, under daylight, or better, artificial light, to note their number, their motility, their size, form and spore formation, their general arrangement, their reactions to specific serums and to vital stains, etc. But for more exact study of their structure they must be stained in a dried film preparation on a glass slide or a cover-glass or when in tissues, in sections.

Elimination of Foreign Organisms from Preparations.—Since germs are present in the air, in dust, in tap-water, on our bodies, clothes and on all surrounding objects, it follows that when we begin to examine substances for microbes the first requisite is that the materials we use, such as staining fluids, cover-glasses, etc., should be practically free from organisms, both living and dead, otherwise we may not be able to tell whether those we detect belong originally in the substances examined or only in the materials we have used in the investigation. Therefore, all solutions are filtered and all apparatus thoroughly cleaned and when necessary sterilized.

Examination of Bacteria in the Hanging Drop.—For this examination special slides and methods are desirable. The slide used is one in which there is ground out on one surface a hollow having a diameter of about $\frac{1}{2}$ inch (Fig. 13). According to the purpose for which the hanging drop is to be studied, sterilization of the slide and cover-glass may or may not be necessary.

Fig. 13.—Hollow slide with cover-glass.

The technic of preparing and studying the hanging drop is as follows: The surface of the glass around the hollow in the slide is smeared with a little vaselin or other inert oil. This has for its purpose both the sticking of the cover-glass to the slide and the prevention of evaporation in the drop placed in the little chamber which is to be formed between the cover-glass, when placed over the hollow, and the slide.

If the bacteria to be studied are in a fluid, we place a platinum loopful upon the center of the cover-glass and, to avoid drying, immediately invert it by means of a slender pair of forceps over the hollow in the slide, being very careful to have the drop over the center of the cover-glass. The cover-glass is then pressed on the slide so as to spread the vaselin and make a perfect seal.

If the bacteria are growing on solid media, or are obtained from thick pus or tissues from organs, they are mixed with a suitable amount of bouillon or sterile physiological salt solution[1] either before or after being placed upon the cover-glass.

In studying living bacteria to determine only their grouping and motion we may use less magnification than for studying other characteristics. In studying unstained bacteria and tissues we shut off as large a portion of the light with our diaphragm as is compatible with distinct vision, and thus favor contrasts which appear as lights and shadows, due to the differences in light transmission of the different materials under examination. It is necessary to remember that they are seen with difficulty, and that we are very apt, unless extremely careful in focussing, to allow the lens to go too far, and so come upon the cover-glass, break it, destroy our preparation, and, if examining pathogenic bacteria, infect the lens. This may be avoided by first finding the hanging drop with a low-power lens and then centering it. The edge of the drop is focussed more easily than the center. The lens of higher magnification is now very gradually lowered, while watched from the side until the tip of the objective throws a shadow on the cover-glass. Then, with eye to eye-piece, focus up carefully until organisms come into view.

Hanging Mass or Hanging Block Cultures.—In order to study the morphology and manner of multiplication of individual microörganisms to better advantage than in the hanging drop, we have used hanging masses of agar, made by placing a large platinum loopful of melted agar on a sterile cover-glass and allowing it to harden, protected from contamination. The organisms are placed on the free surface of this mass which is then inverted over a hollow slide and studied as in a hanging drop.

Hill devised the following procedure: Melted nutrient agar is poured into a Petri dish to a depth of about $\frac{1}{8}$ to $\frac{1}{4}$ inch. When cool, a block is cut out about $\frac{1}{4}$ inch square. The block is placed, under surface down, on a slide and protected from dust. A very dilute suspension of the growth to be examined is then made in sterile bouillon and spread over the upper surface of the block. The slide and block are then put in the incubator for ten minutes to dry slightly. A clean cover-slip is now placed on the agar block in such a way as to avoid large air bubbles. The slide is then removed. With the aid of a platinum loop a drop or two of melted agar is run along each side of the block to fill any angle between it and the cover-glass. After drying in the incubator for five minutes it is placed over a hollow slide and sealed with paraffin.

We consider the hanging-mass method better than the hanging block in many instances, because in the former method no pressure is exerted on the bacteria, and more oxygen is allowed them.

Film Preparation (Spread, Smear).—Film preparation is made as follows: A very small amount of the blood, pus, discharges from mucous membranes, cultures from fluid media, or other material to be examined is removed, usually by means of a sterile swab or platinum

[1] Physiological salt solution is usually 0.8 per cent. NaCl in distilled water.

loop, and smeared undiluted in an even, thin film over a perfectly clean,[1] thin cover-glass or slide. From cultures on solid media, however, on account of the abundance of organisms in the material, a little of the growth is diluted by adding it to a small loopful of filtered or distilled water, which has been previously placed on the glass slide. It is best to add to the drop just enough of the culture to make a perceptible cloudiness. Blood films may be made either by the cover-glass or the slide method.

To make a cover-glass preparation, two square, very thin (hence flexible) cover-glasses are cleaned. Holding one with thumb and index fingers by opposite corners, the tip of a drop of blood obtained by needle puncture of finger or lobe of ear is made to touch the center of the cover-glass, and the second clean cover-glass held similarly is allowed to fall upon the first one in such a manner that the corners do not coincide. The blood droplet spreads by capillarity into a thin film, which is a sign to pull the two covers apart in the plane in which they lie; good results depend upon cleanliness, rapidity and success in sliding the two covers apart.

To make a slide film, the tip of the exuded blood drop is made to touch one slide near one end, and the edge of the second slide, held at an acute angle to the first one, is made to bisect the drop, which will spread at the point of contact by capillarity across the slide. Upon pulling the second or spreading slide over the first slide, never changing the angle and applying gentle pressure, a thin layer of blood suitable for examination will be formed. A slide made in this manner should be dried immediately by agitation in the air. It may then be fixed and stained in various ways.

Milk films, after fixation, are cleared of fat by means of ether, xylol or alkaline solution.[2]

The film either is dried thoroughly in the air and then fixed with heat or any chemical fixative, or it may be placed in any of the fixatives while still moist. The usual fixatives are methyl alcohol, absolute ethyl alcohol, Zenker's solution, etc. (see pp. 90 and 91).

When fixed with heat, the glass is held by any one of the several kinds of forceps commonly used and is passed three times by a rather slow movement through the Bunsen or alcohol flame.

[1] To render new cover-slips clean and free from grease, the method recommended by Gage is useful: Place in following solution overnight:

Bichromate of potash ($K_2Cr_2O_7$)	200 gm.
Water, tap or distilled	800 c.c.
Sulphuric acid	1200 c.c.

The bichromate is dissolved in the water by heating in an agate kettle; the sulphuric acid is added very slowly and carefully on account of great heat developed. After cooling, it is kept in a glass vessel. It may be used more than once.

Glasses are removed the next morning and cleansed in running tap-water until the yellow color disappears. They are then placed in ammonia alcohol until used. When used, wipe with soft, clean linen or cotton cloth. If old cover-slips are used, boil first in 5 per cent. sodium carbonate solution.

Another procedure is, after washing with soap and water and rinsing in water, to soak the cover-glasses in alcohol, then wipe with soft linen, then place in a Petri dish, and heat in the dry sterilizer for one hour at 200° C. to burn off fatty substances. The heating may be done by holding the cover-glass in the flame sufficiently to heat thoroughly without softening. A cover-glass is not clean when a drop of water spread over it does not remain evenly distributed, but gathers in droplets.

[2] One-half or 1 per cent. sodium hydrate.

The film thus prepared is usually stained either by the simple addition of a solution of a coal-tar dye, for from a few seconds to five minutes, or by one of the more complicated special stains described later. When the stain is to be hastened or made more intense, the dye is used warm.

The cover-glass or slide, with the charged side uppermost, may either rest on the table or be held by some modification of Cornet's forceps. When the solution is to be warmed, the cover-glass may be floated, smeared side down, upon the fluid contained in a porcelain dish resting on a wire mat, supported on a stand; or the solution may be poured on the glass which may then be held over the flame in the Cornet forceps. If a slide is used, it is simply inserted in the fluid or covered by it. The fluid both in the dish and on the slide should be carefully warmed so as to steam without actually boiling. The slide should be kept completely covered with fluid.

All stains should be filtered and controlled for presence of microbes before use.

After staining the film, the cover-glass or slide is grasped in the forceps and thoroughly but gently washed in clean water and then dried, first between layers of filter paper and then in the air or high over a flame. If a cover-glass has been used, a drop of balsam or water is placed on a glass slide and the cover-glass put upon it with the film side down. Films made on slides are usually unmounted. Cedar oil is added at the time of examination with the oil-immersion lens and washed off with xylol immediately after.

Burri's India-ink Method of Demonstrating Bacteria.[1]—In 1907, 1908 and 1909 Burri recommended the following method for isolating and studying single bacteria cells: A solution of India ink (flüssige Perltusche) in water 1 to 10 (better 1 to 4) is sterilized in test-tubes in the autoclave for fifteen minutes. A small drop of this ink is mixed carefully with a drop of the fluid to be examined. If cultures from isolated cells are desired, the culture should first be diluted so that a drop of the mixture of ink and bacteria contains *presumably* a single organism; then drops of the mixture are placed in rows upon nutrient agar plates and each drop examined microscopically after placing a cover-glass over it. The single bacteria can be easily found. To study further the plate may be placed at the proper temperature or the cover-glass may be removed from any drop and the portion containing the single organism may be transplanted to another medium. If the bacteria are to be examined immediately, a drop of the mixture (ink plus undiluted bacterial fluid) is allowed to dry upon a glass slide and then examined under an oil-immersion lens. The bacteria appear a brilliant white upon a dark field, particles of the ink surrounding the organisms like a capsule. This method is especially applicable for the demonstration of such organisms as the Tr. pallidum which have poor staining qualities and a low index of refraction.

Burri also combined with this method the staining of the bacteria. Dorner,[2] one of Burri's associates, has combined the staining of spores with this method.

Stains and Staining Methods for Microörganisms.—As applied in bacteriology, dyes are used for four purposes: to render bacteria visible, to show structure, to indicate chemical nature and to influence growth. Most organisms, with the exception of the acid-fast group, are stained easily with simple dye solutions. Special methods are necessary for

[1] Das Tuscheverfahren als einfaches Mittel, etc., Jena, 1909.

[2] Landworlsch: Jahrbuch d. Schweiz, 1922.

the staining of spores, capsules and flagella. Protozoa stain in general as do higher animal cells. The protoplasm of mature bacteria reacts to stains much as does the nuclear chromatin of animal cells, though the intensity of the staining varies somewhat with the species and the condition of growth, such as the age, the media and so on.

Coal-tar Dyes.[1]—A dye may be defined as an organic compound containing benzene rings to which are attached chromophoric and auxochromic groups. The chromophoric group gives to the dye its color property while the dyeing property is due to the auxochromic group, which may be either acidic or basic in character. Dyes are either "basic" or "acid," depending upon the auxochromic group. Basic dyes are regarded as nuclear stains, since they color the nuclear chromatin of both cells and bacteria. The acid dyes are used by the histologist chiefly as contrast (plasma) stains. The basic dyes are usually employed as salts of a colorless acid (as hydrochloric); the acid dyes as sodium or potassium salts.

The following are the most commonly used coal-tar dyes:

Blue stains—methylene blue, thionin blue (give the best differentiation).
Red stains—basic fuchsin, safranin.
Brown stain—Bismarck brown (weak, may be used as counter-stain).
Green stain—methyl green.
Pink stain—eosin (weak; may be used as counter-stain), Rose bengal.[2]
Violet stains—methyl violet, gentian violet, crystal violet (most intense stains; may overstain).

These dyes are all more or less crystalline powders, and while some are definite chemical compounds others, owing to difficulties encountered in the manufacture, are mixtures. For this reason various brands are met with in the market. Many of the commonly used dyes have been placed on a certification basis by the Commission on Standardization of Biological Stains. For reliable results only dyes certified by the Commission should be used in the preparation of staining solutions.[3]

Stock saturated alcoholic solutions from which staining solutions are made may be kept on hand. These solutions are made by shaking an excess of the dye with 95 per cent ethyl alcohol. Enough of the dye is added so that a sediment remains after mixing well and allowing to stand for twenty-four hours.

Since, as noted above, some dyes may be mixtures of two or more dyes having a different solubility in alcohol, the sediment from a saturated alcoholic solution should not be used in preparing a further quantity of the solution.

The dilution used for staining with single stains may be prepared by adding to distilled water sufficient of the saturated alcoholic solution, so that one can just see through the solution. Usually 1 part in 10 is sufficient. In some cases a more concentrated solution is required. The dilute solutions do not keep well and must be made fresh when necessary.

A staining solution prepared by dissolving a given amount of the dry dye of known dye content in a suitable solvent is probably more satisfactory than the use of saturated alcoholic solutions.

General Observations on the Principles of Staining Microörganisms.—The staining of microbes is not to be considered simply as a mechanical

[1] For a good description of the composition and action of the various stains, see A. B. Lee's Microtomist's Vade-Mecum, 9th ed., Philadelphia, P. Blakiston's Son & Co., 1928.

[2] Recommended as bacterial stain, especially for slime formers, by H. J. Conn (Jour. Bacteriol., 1921, **6**, 253).

[3] Biological Stains (1925), published by the Commission on Standardization of Stains (H. J. Conn, Chairman, Geneva, N. Y.), gives the reports of the Commission, also data as to composition of stains. Stain Technology, a journal published by the Commission, lists the stains which have been certified.

saturation of the cell body with the dye, in which the latter is dissolved in the plasma. It is supposed that a chemical combination between the dye substance and parts of the organism may also occur.

The dependence of the staining process upon the solvent condition of the dye is shown in the following observations:

1. Entirely water-free, pure alcoholic dye solutions do not stain well.

2. Absolute alcohol does not decolorize dehydrated bacteria, while diluted alcohol is an active decolorizing agent. The compound of dye substance and plasma is, therefore, less soluble in pure alcohol.

3. The more completely a dye is dissolved the weaker is its staining power. For this reason pure alcoholic solutions are inactive; and the so-called weak dye solutions to which strong dye solvents have been added are limited in their action on certain bacteria in which the dye substance is closely united. This is the principle of Neisser's stain[1] for diphtheria bacilli, viz., acetic-acid-methylene-blue solution.

On the other hand, the addition of alkalis to the dye mixture renders the solvent action less complete and the staining power more intense. According to Michaels, however, in Löffler's methylene-blue solution the role of the alkali is purely of a chemical nature, by which it converts the methylene blue into methylene azur (azur II).

The dependence of the staining process upon the nature of the microbe is exhibited in the following facts:

Certain microbes stain easily, others with difficulty. To the latter belong, for example, the tubercle bacillus and lepra bacillus. Spores and flagella also stain with difficulty. The easily stained objects require but a minimum of time to be immersed in a water solution, while the others must be stained by special dyes with or without the aid of outside influences (heat, mordants, etc.). The difficultly stained objects are at the same time not easily decolorized. The explanation of the resistance which these bacteria show to staining as well as to decolorizing agents is to be sought in two ways: either on the assumption that they possess a difficultly permeable or a resisting envelope, or that they have a special chemical constitution. The latter hypothesis holds good in regard to flagella, spores and most bacteria. In some instances, doubtless, both of these causes, viz., resistant envelope and chemically different constitution, work together to produce the above mentioned results.

Selective staining properties, whereby certain species of organisms are exclusively or rapidly and intensely stained by certain dyes; have repeatedly been observed. Of the greatest practical importance in this respect is the *Gram stain* (see p. 82 and Chapter VI), used for the differential diagnosis of many species of bacteria. Other species, again, are at one time stained and at another decolorized by Gram; thus pyocyaneus is stained only in young individuals. Previous heating or extraction with ether does not prevent the action of Gram's stain, but treatment with acids or alkalies renders it impossible. Bacteria so treated, however, after one hour's immersion in Löffler's mordant regain their property of staining with Gram.

Mordants and Decolorizing Agents.—We have already noted that the protoplasm of unrelated microbes may respond differently to the several dyes. There is, however, seldom any difficulty in selecting a dye which will stain sufficiently to make microbal cells in pure cultures distinctly visible. When the microbes are imbedded in tissue or mixed in a film with blood or pus, it is frequently difficult to prevent the stain from so acting on the tissue or pus elements as to obscure the organisms.

[1] Ztschr. f. Hyg., 1897, 24, 443.

Various methods are then employed to stain the germs more intensely than the tissues or to decolorize the tissue more than the organisms. Heating, the addition of alkali to the staining fluid and prolonging the action of the dyes increase the staining properties. We regulate these so as to give the best results. We also use mordants; that is, substances which fix the dye (whether physically or chemically is still to be decided) to the bacterial cell, such as anilin oil or solutions of carbolic acid and metallic salts. The decolorizing agents used chiefly are mineral acids, vegetable acids, diluted alcohols, various oils and hot water.

Formulæ of the More Generally Used Special Staining Mixtures.—Alkaline Methylene Blue (Löffler's Methylene-blue Solution).—Saturated alcoholic solution of methylene blue, 30 c.c.; caustic potash in a 0.01 per cent. solution, 100 c.c. The alkali not only makes the cell more permeable, but also increases the staining power by liberating lower homologs (making it a polychrome stain) from the dye, which have an affinity for chromatin and certain cell granules. It is therefore a particularly good stain for the diphtheria bacillus.

Films stained two to five minutes, heated if more intense stain is desired.

Many quicker methods have been proposed for preparing a polychrome methylene blue, the chief change being the use of heat (Nocht, 1898, Leishman 1901, Wright, 1906, Goldhorn, 1909). Most of these have been made in connection with eosin combinations.

Methylene Blue (Polychrome) and Eosin Mixtures (Tetrachrome Stain MacNeal).—These staining mixtures introduced by Wright, Romanowsky (1891) Nocht, and others have been used chiefly for the staining of animal cells (blood cells, protozoa) but they are also useful in differentiating bacteria and other germs, especially in differentiating those organisms that take ordinary stains faintly, such as the spirochetes. They are fine differential stains for chromatin. Many modifications have been proposed.

MacNeal[1] has called attention to the fact that the essential dyes of this staining mixture are four in number rather than three as Giemsa had maintained. These four dyes are methylene azur, methylene violet, methylene blue and eosin. MacNeal and Schule (1913) recommended a mixture of the following composition: water soluble eosin, 1 gm.; medicinally pure methylene blue, 1 gm.; methylene azure, recrystallized, 0.6 gm.; methylene violet, recrystallized, 0.2 gm.; pure methyl alcohol (Merck's reagent), 1000 c.c. This should be kept in two solutions, the eosin in 500 c.c. methyl alcohol, in one, and the rest in the other. They should be mixed in equal parts at the time of use . They keep well for about two years. Crude products of methylene violet may be substituted for the purified product, but must be used in double the quantity.

A finely ground mixture of these four dyes has recently been put on the market in this country, which is just as satisfactory and much cheaper than the foreign preparation.

Jenner's Stain.[2]—Equal parts of eosin (water soluble) 1.2 per cent. aqueous solution and methylene blue (medicinal) 1 per cent. aqueous solution; rinse and allow to stand twenty-four hours. The dark coarse granular precipitate with a metallic luster is separated by filtering through fine filter paper (C. P.) and washed with water until filtrate runs clear. For the stain 0.5 gm. of the dried precipitate is dissolved in 100 c.c. methyl alcohol. The stain is poured on the unfixed film (dried in air) and allowed to remain for one to two minutes, then washed off with distilled water. The film is dried and is ready for examination.

[1] MacNeal, W. J.: Jour. Am. Med. Assn., 1922, **78**, 1122; Jour. Inf. Dis., 1925, **36**, 538.
[2] Lancet, 1889.

Leishman Stain.[1]—*Solution A.* To a 1 per cent. solution of medicinally pure methylene blue in distilled water add 0.5 per cent. sodium carbonate and heat at 65° C. for twelve hours, then allow it to stand ten days at room temperature. *Solution B.* Eosin extra B–A (Grübler) 0.1 per cent. solution in distilled water.

Mix Solution A and B in equal amounts and allow to stand six to twelve hours, stirring at intervals. Filter and wash the precipitate thoroughly. Collect, dry and powder it. 0.15 gram is dissolved in 100 c.c. of pure methyl alcohol to form the staining solution. It keeps perfectly for at least five months. To stain, cover the dried but unfixed film of blood with the staining solution. After thirty to sixty seconds add about an equal amount of distilled water. Allow this mixture to act for five minutes. Wash in distilled water for about one minute, examining the specimen mounted in water under the microscope. Blot, dry thoroughly, mount in balsam, or preserve the specimen as an unmounted film.

Wright's Stain.[2]—One per cent. methylene blue (alcohol rectified) and 0.5 per cent. sodium carbonate are mixed and placed in a steam sterilizer for one hour. When cold, 0.1 per cent. solution of extra B-A eosin (500 c.c. eosin to 600 c.c. methylene-blue solution) is added until the mixture becomes purplish, and a finely granular black precipitate appears. This precipitate is filtered off and dried without being washed. A saturated solution of this is made in pure methyl alcohol. This is filtered and then diluted by adding to 40 c.c. of it 10 c.c. of methyl alcohol. In using, a few drops are placed on the film for a minute; then water is dropped on until a greenish iridescence appears. The stain then remains on for two minutes; then is washed off with distilled water, allowing a little to remain on until differentiation is complete. Dried with filter paper. Haden[3] says that precipitate on film is due to excess of alkali and it may be avoided by use of buffer solution of KH_2PO_4 or Na_2HPO_4 with a pH 6.4 (6–6.6) for diluting stain instead of distilled water.

Giemsa's Method.[4]—Smears are fixed in neutralized methyl alcohol for one minute. There are several variations of Giemsa's method. One of them is given here:

Azur II—Eosin	3.0 gm.
Azur II	0.8 "
Glycerin (Merck, chem. pure)	250.0 c.c.
Methyl alcohol (chem. pure)	250.0 "

Both glycerin and alcohol are heated to 60° C. The dyes are put into the alcohol and the glycerin is added slowly, stirring. The mixture is allowed to stand at room temperature overnight, and after filtration is ready for use.

The solution is prepared ready for use by certain firms.

One drop of the stain to every cubic centimeter of distilled water, made alkaline by the previous addition of 1 drop of a 1 per cent. solution of potassium carbonate to 10 c.c. of the water, is poured over the slide and allowed to stand for one-half to three hours. The longer time brings out the structure better, and in twenty-four hours well-made smears are not overstained. After the stain is poured off, the smear is washed in running tap-water for one to three minutes, and dried with filter paper. If the smear is thick, the organisms may come out a little more clearly by dipping in 50 per cent. methyl alcohol before washing in water; then the washing need not be so thorough. By this method of staining, the cytoplasm of protozoa stains blue and the nuclear substance a blue red or azur. Young bacteria usually take a dark purple stain, and their metachromatic granules an azur.

[1] British Med. Jour., 1901, p. 635; 1902, p. 757.
[2] Jour. Med. Research, 1902, **2** (new series), 138.
[3] Jour. Lab. and Clin. Med., 1923, **9**, 64.
[4] Deutsch. med. Wchnschr., 1905, **31**, 1026.

The Eosin-methylene-blue Method Recommended by Mallory for Tissues may be Used for Smears as Follows.—The smears are fixed in Zenker's solution for one-half hour; after being rinsed in tap-water they are placed successively in 95 per cent. alcohol + iodin, one-quarter hour; 95 per cent. alcohol, one-half hour; absolute alcohol, one-half hour; eosin solution, twenty minutes, rinsed in tap-water; methylene-blue solution, fifteen minutes, differentiated in 95 per cent. alcohol from one to five minutes; and dried with filter paper.

Fuchsin and Phenol (Carbolfuchsin, Ziehl-Neelsen Solution).—Distilled water, 100 c.c.; carbolic acid (crystalline), 5 gm.; 95 per cent. alcohol, 10 c.c.; fuchsin, 1 gm.; or it may be prepared by adding to 90 c.c. of a 5 per cent. watery solution of carbolic acid, 10 c.c. of a saturated alcoholic solution of fuchsin.

The last two methods, combined with heating, are used to stain spores and certain resistant bacteria as the tubercle bacilli and other "acid resisters," so that they retain their color when exposed to decolorizing agents (see below).

Methylene Blue and Phenol (Carbol Methylene Blue, Kühne).—1.5 gm of methylene blue, 10 gm. of absolute alcohol, and 90 c.c. of a 5 per cent. solution of carbolic acid.

Phenol, in order to intensify the action, may be added to various dyes, usually in the proportion of 1 part of a saturated alcoholic solution of the dye to 9 parts of a 5 per cent. solution of phenol. Thus, we may have carbolgentian violet, carbol thionin, and so on.

Koch-Ehrlich Anilin Water Solution of Fuchsin or Gentian Violet is prepared as follows: To 98 c.c. of distilled water add 2 c.c. anilin oil, or more roughly but with equally good results, pour a few cubic centimeters of saturated anilin oil into a test-tube, then add sufficient water nearly to fill it. In either case the mixtures are thoroughly shaken and then filtered into a beaker through moistened filter paper until the filtrate is perfectly clear. To 75 c.c. of the filtrate (anilin water) add 25 c.c. of the saturated alcoholic solution of either fuchsin, methylene blue, or gentian violet, or add the alcoholic solution until the anilin water becomes opaque and a film begins to form on the surface.

Gram's Stain.[1]—Among the differential methods of staining, Gram's method is the most widely applicable. The solutions employed are:

(*a*) Gram's stain (initial stain), a solution of a pararosanilin dye (gentian violet, methyl violet, crystal violet, Victoria blue) in distilled water with or without the addition of anilin oil or phenol. Crystal violet, since it is a definite chemical compound and may be obtained pure as a certified dye, is probably the most suitable. It should be noted that the rosanilin dyes are not suited for this purpose.

(*b*) Gram's iodine (Lugol's solution), composed of iodine, 1 gm., potassium iodide, 2 gm., distilled water, 300 c.c. This solution should be kept in dark bottles, since when exposed to light it decomposes, forming acid. The use of the solution in this condition has a tendency to cause Gram-positive bacteria to show a Gram-negative reaction.[2] An alkaline iodine solution overcomes this difficulty.[3]

(*c*) Decolorizer: Either 95 per cent alcohol or acetone or a mixture of equal parts of the two[4] may be used. Acetone is a much more rapid decolorizing agent than alcohol and, while it must be used with caution, it gives very satisfactory results.[5] The dilution of either decolorizing agent with water increases the power of decolorizing the Gram-positive bacteria.[6] It is advisable not to

[1] For a general discussion of Gram's Stain, see Churchman, J. W.: The Newer Knowledge of Bacteriology and Immunology, 1928, p. 19.

[2] Scheppe and Constable: Jour. Lab. and Clin. Med. Jour., 1923, **8**, 757.

[3] Kopeloff and Beerman: Jour. Inf. Dis., 1922, **31**, 480.

[4] Kopeloff and Cohen: Stain Technology, 1928, **3**, 64.

[5] Stern and Stern: Am. Jour. Pub. Health, 1928, **18**, 174.

[6] Burke, V.: Jour. Bacteriol., 1922, **7**, 159.

use the decolorizer more than once and to remove excess water from the slide before application.

(*d*) Counter-stain: Dilute carbol-fuchsin (1 to 20), safranin 0 (2 per cent. aqueous solution) or Bismarck brown (0.1 per cent. solution) may be used.

Practically all bacteria, with the exception of the acid-fast group are stained by the initial stain. The addition of the iodine solution causes a fixation of the stain, as regards solubility in alcohol or acetone, in the case of Gram-positive bacteria. No such fixation occurs in the Gram-negative bacteria. This fixation may be of a physical or chemical nature or, as is most probable, a combination of both. Many observers look upon the reaction as purely chemical,[1] while others, notably Benians,[2] regard it as purely physical. Based upon the fact that Gram-negative bacteria may be stained with an alcoholic solution of the dye-iodine complex while the Gram-positive bacteria are not so stained Burke[3] corroborates the conclusions of Benians. The Gram reaction of an organism may be influenced by the hydrogen-ion concentration of the medium[4] or the bacterial proteins. Since the latter changes with age, this probably accounts for the variation of the Gram reaction in young and old cultures of the same organism.

Various workers have suggested the Gram-positivity as due to unsaturated fatty acids having high affinity for iodine;[5] to the degree of dispersal of nucleoproteins;[6] to the compound bacterial lipoproteins of unsaturated fatty acids, or to the formation of a dye-iodine-protein compound which is insoluble in the decolorizing agent.[7] The action of the mordant, aside from the formation of a dye-iodine complex, may be one of oxidation.[8]

Many modifications of the Gram stain have been suggested.[9] Those of Burke and Kopeloff and Beerman give satisfactory results. In our hands the method of Burke, using the alkaline iodine solution suggested by Kopeloff and Beerman, gives very good contrast with a minimum of extraneous matter remaining in the smear. The following procedure may be suggested: The smear, which should be thin, is fixed with heat and flooded with the initial stain (1 per cent aqueous solution of crystal violet) to which is added 4 to 5 drops of 5 per cent water solution of sodium bicarbonate. Staining is continued for two minutes in the cold. The excess stain is poured off, the slide washed with water and iodine solution (iodine, 2 gm., normal sodium hydrate, 10 c.c., water, 90 c.c.) poured on the slide. After acting for two minutes the iodine solution is removed, the slide washed with water and the excess of water removed by blotting. The slide must not be allowed to become dry. The decolorizer (acetone, or acetone-alcohol, 50 per cent each) is applied drop by drop until the dye no longer flows from the smear; usually about thirty seconds is sufficient. The slide is allowed to air dry and then the counterstain is applied (carbol-fuchsin, 1 to 20, for thirty seconds). Wash, and blot dry. The Gram-positive bacteria will be stained a deep blue, the initial stain having been retained while the Gram-negative bacteria takes the color of the counterstain.

This method is useful in demonstrating the capsule which is seen to surround some bacteria, such as the pneumococcus, and also in differentiating between varieties of bacteria. See Chapter VI for further remarks upon Gram's stain.

The Modifications of Gram's Stain are Many.—One only is given here.

Nicolle's Modification.—Stain cold in carbol-gentian violet one minute; wash in tap-water; stain cold in the iodine mixture one minute; wash in tap-

[1] Deussen, E.: Ztschr. f. Hyg. u. Infectionskrankh., 1918, **85**, 235.
[2] Benians, T. H. C.: Jour. Path. and Bacteriol., 1912, **17**, 199; 1919–1920, **23**, 411.
[3] Burke, V.: Jour. Bacteriol., 1922, **7**, 159.
[4] Stern and Stern: Jour. Bacteriol., 1925, **9**, 463, 479, 491; **10**, 13.
[5] Jobling and Peterson: Jour. Exp. Med., 1914, **20**, 456.
[6] Hottinger, R.: Centralbl. f. Bakteriol., 1916, **76**, 367.
[7] Unna, P. G.: Monatschr. f. prakt. Dermatol., 1887, Suppl. No. 6.
[8] Stern and Stern: Jour. Bacteriol., 1925, **9**, 463, 479, 492; **10**, 13.
[9] Hucker and Conn: Technical Bulletin No. 128, New York State Experimental Station, Geneva, N. Y., 1927.

water; decolorize ten seconds in acetone (1 part) and alcohol (3 parts); wash in tap-water; counter-stain ten seconds in dilute carbolfuchsin (1 to 10).

Stains for Metachromatic Granules.—A good polychrome methylene blue solution, *e. g.*, Löffler's methylene blue, brings out the granules perhaps more differentially than other staining methods that have been recommended. Neisser's method is one of the best of these other methods.

Neisser Stain.—The Neisser stain is carried out by placing the cover-slip smear of diphtheria or other bacilli in solution No. 1 for from two to three seconds, and then, after washing, in No. 2 for from three to five seconds. The bacilli will then appear either entirely brown or will show at one or both ends a dark blue, round body. With characteristic diphtheria bacilli, taken from a twelve to eighteen hours' growth on serum, nearly all will show the blue bodies, while with pseudo types, few will be seen.

The solutions are as follows:

No. 1.—Alcohol (96 per cent.), 20 parts; methylene blue (Grübler), 1 part; distilled water, 950 parts; acetic acid (glacial), 50 parts.

No. 2.—Bismarck brown, 1 part; boiling distilled water, 500 parts.

Staining of Capsules.—Many methods of demonstrating the capsule have been devised. Three only will be given here.

Welch's Glacial Acetic Acid Method is as follows:[1] (1) Cover the preparation with glacial acetic acid for a few seconds; (2) drain off and replace with anilin gentian-violet solution; this is to be repeatedly added until all the acid is replaced; (3) wash in 1 or 2 per cent. solution of sodium chloride and mount in the same. Do not use water at any stage. The capsule stains a pale violet. (See Plate III, Fig. 15.)

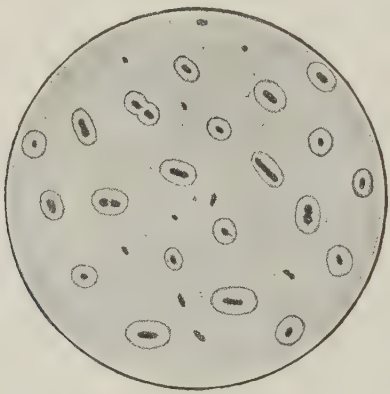


Fig. 14.—Capsule stain by Hiss's method. Rhinoscleroma bacillus. × 1000. (Thro.)

Hiss's Copper Sulphate Method (Fig. 13).[2]—The organisms are grown, if possible, on ascitic fluid or serum media. If not, the organisms should be spread on the cover-glass mixed with a drop of serum, or better, with a drop of one of the diluted serum media. Dry in the air and fix by heat.

The capsules are stained as follows: A 5 per cent. or 10 per cent. aqueous solution of gentian violet or fuchsin (5 c.c. saturated alcoholic solution gentian violet to 95 c.c. distilled water) is used. This is placed on the dried and fixed cover-glass preparation and gently heated for a few seconds until steam arises. The dye is washed off with a 20 per cent. solution of copper sulphate (crystals). The preparation is then placed between filter paper and thoroughly dried. (See Plate III, Figs. 14 and 16.)

[1] Johns Hopkins Hospital Bull., 1892, **3**, 81. [2] Jour. Exper. Med., 1905, **6**, 317.

Huntoon's Method[1] (Fig. 1, p. 31).—A 3 per cent. solution in distilled water of nutrose (sodium casinate) is cooked for one hour in an Arnold and tubed unfiltered after adding 0.5 per cent. carbolic. The organisms to be stained are mixed with a drop of this solution of nutrose, spread in thin film on a glass slide and dried in air, not fixed. The stain is as follows: Carbolic acid (2 per cent. aqueous solution), 100 c.c.; acetic acid (1 per cent. aqueous solution), 1 c.c.; lactic acid (concentrated), 0.5 c.c.; carbol fuchsin, 1 c.c.; basic fuchsin (saturated alcoholic solution), 1 c.c. The stain is kept on the film for thirty seconds, washed in water and dried.

Staining Spores.—We have already noted that during certain stages in the growth of a number of bacteria, spores are formed which refuse to take up color when the bacteria are stained in the ordinary manner. Special methods have been devised for causing the color to penetrate through the resistant spore membrane.

In the simplest method a cover-slip after having been prepared in the usual way is covered with Ziehl-Neelsen's (p. 82) carbol-fuchsin solution and held over the Bunsen flame until the fluid steams. This is continued for one or two minutes. It is then washed and dipped in a decolorizing acid solution, such as a 2 per cent. alcoholic solution of nitric acid, or a 1 per cent. solution of sulphuric acid in water, until all visible color has disappeared, then it is washed in running water and dipped for one-half minute in a saturated watery solution of methylene blue. The bodies of the bacilli are blue and the spores red. This same method is also used for staining acid-fast bacilli. Sometimes the spores refuse to take the stain in this manner. We then can try other methods.

Moeller's Method.—The prepared cover-slip is held for two minutes in chloroform, then washed off in water and placed from one-half to three minutes in a 5 per cent. solution of chromic acid,[2] again washed off in water, and now stained by carbol-fuchsin, which is steamed for several minutes. The staining fluid is then washed off and the preparation decolorized in a 3 per cent. solution of hydrochloric acid or a 5 per cent. solution of sulphuric acid. The preparation is finally stained for a minute in methylene-blue solution. The spores will be red and the body of the cells blue. The different spores vary greatly in the readiness with which they take up the dyes, and we have, therefore, to experiment with each variety as to the length of time it should be exposed to the maceration of the chromic acid. Even under the best conditions it is almost impossible to stain some spores.

Huntoon's Spore Stain.—4 gm. *acid* fuchsin (Grübler) dissolved in 50 c.c. 2 per cent. aqueous acetic acid.

2 gm. methylene blue (Grübler) dissolved in 50 c.c. 2 per cent. aqueous acetic acid.

Mix the two solutions, shake and set aside for fifteen minutes. Heavy precipitate results. Filter mixture through well-moistened filter paper. Use the filtrate for staining. Reddish-purple filtrate will keep for several weeks. Refilter if precipitate appears.

To stain, make a rather thick smear, preferably from an agar slant. Cover smear with dye and steam one minute. Wash in water. Film appears bright red. Dip slide in dilute solution of sodium carbonate (7 or 8 drops saturated solution in tumblerful of water). When film turns blue, rinse immediately in water. Dry and examine. Spores are stained red, the body of the bacillus is stained blue. (Plate II, Fig. 22, shows stained spores.)

May[3] has suggested a method of pretreating spores with chromic acid (1 per

[1] Jour. Am. Med. Assn., 1914, **62**, 18, 1397; also Proc. Soc. Bacteriol., December, 1916.

[2] Bittman in our laboratory obtained better results by steaming the chromic acid.

[3] Stain Technology, 1926, **1**, 105.

cent.) and concentrated ammonia. This treatment is followed by staining with carbol-fuchsin, decolorizing with sulphuric acid (1 per cent.) and counterstaining with methylene blue.

Dorner's method[1] consists in suspending the bacteria in hot carbol-fuchsin, making a smear of the suspension together with a saturated solution of nigrosin. The spore is stained red, while the vegetative forms are colorless against darkened background.

Burke[2] has shown that dead spores will be stained by direct steaming with carbol-fuchsin; live spores remain unstained.

Staining Acid-fast Bacteria.—Ziehl-Neelson Method.—This is the usual stain with carbol-fuchsin given in the general method under "staining spores," above. (See Plate VI.)

Hermann Stain.—A, crystal violet 3 per cent. solution in alcohol; B, ammonium carbonate 1 per cent. solution in water; mix 1 part of A with 3 parts of B just before using; steam the fixed films three minutes, decolorize with 10 per cent. nitric acid, wash in alcohol, and counter-stain in Bismarck brown. (See Plate VI.)

Baumgarten's Method for Differentiating the Tubercle Bacillus from the Bacillus of Leprosy.—The fixed films are stained in dilute alcoholic fuchsin five minutes; then decolorized for twenty seconds in a mixture of 95 per cent. alcohol, 10 parts and nitric acid 1 part; washed in water; counter-stained in methylene blue, washed in water, dried, and mounted. The tubercle bacilli do not take up the dilute alcoholic fuchsin as readily as do the leprosy bacilli, therefore the former are stained a faint blue, the latter a red.

Pappenheim's Method of Differentiating Between the Tubercle and the Smegma Bacillus.—Films fixed by heat in usual way; stained with hot carbol-fuchsin two minutes; dye poured off; without washing film, it is covered with the following mixture: Rosalic acid, 1 gm.; absolute alcohol, 100 c.c.; methylene blue added to saturation; when these are mixed, glycerine, 20 c.c., is added. This mixture is poured on the film and off slowly 4 or 5 times. Finally the film is washed in water and dried. The smegma bacilli are decolorized, the tubercle bacilli are red.

Konrich Method.[3]—Stain the fixed films with steaming carbol-fuchsin for one to two minutes; rinse with water; decolorize with 10 per cent. aqueous solution of sodium sulphide for one-half to two minutes; rinse with water; counter-stain with malachite green—50 c.c. saturated aqueous solution in 100 c.c. distilled water—for one-fourth to one-half minute.

Cooper's Method.[4]—The stain is prepared by mixing 3 c.c. of 10 per cent. solution of sodium chloride with 100 c.c. carbol-fuchsin. This stain is stable if kept at incubator temperature, but precipitates at room temperature.

Rapid Method.—Flood the slide with fresh stain; steam for four minutes and allow the slide to cool until the precipitate forms. This takes place in about two minutes and may be hastened by gently blowing on the slide. The precipitate *must* be allowed to form, for on it depends the success of the method. Wash with tap water, decolorize one to ten minutes in acid-alcohol (5 cc. of nitric acid, specific gravity, 1.42, to 95 c.c. of 95 per cent ethyl alcohol); wash again, counterstain with 1 per cent brilliant green in 1 to 10,000 sodium hydroxide for one minute, wash, dry and examine.

Twenty-four-hour Method.—The staining is carried out in the incubator and in the morning the jars containing stain and slides are allowed to stand at room temperature until precipitation takes place, or, better still, in the ice-box. The latter has the advantage of speed as the precipitation is complete in one-half

[1] Jahrb. d. Schwiez., 1922, **36**, 5951; Le Lait, 1926, **6**, 8.
[2] Jour. Inf. Dis., 1923, **32**, 433.
[3] Deutsch. med. Wchnschr., 1920, **46**, 741.
[4] Arch. Path. and Lab. Med., 1926, **2**, 382.

hour. Good slides are obtained in from fourteen to twenty hours' staining. The decolorization and counterstaining are the same as for the rapid method.

Staining Flagella.[1]—For the demonstration of flagella, which are possessed by all motile bacteria, we are indebted first to Löffler. The staining of bacterial flagella well is one of the most difficult of bacteriological procedures. In all methods successively young (twelve-to-eighteen-hour) cultures on elective media should be chosen. With typhoid bacilli and similar varieties enough of the culture to produce slight cloudiness is placed in a few cubic centimeters of filtered tap-water in a test-tube. This may be used immediately, or allowed to stand in the thermostat at blood heat for from one to two hours to permit slight development. A tiny drop of this rather thin emulsion is allowed to spread with as little manipulation as possible over the cover-glass so that it may dry quickly. This latter point seems to be the important one, since slow drying allows the bacteria to shed their flagella.

Bunge's Modification of Löffler's Method is carried out as follows: Cover-glasses which have been most carefully cleaned are covered by a very thin smear. After drying in the air and passing three times through the flame, the smear is treated with a mordant solution, which is prepared as follows: To 3 parts of saturated watery solution of tannin add 1 part of a 25 per cent. solution of ferric chloride. This mordant should be allowed to stand for several weeks before using. After preparing the cover-slip with all precautions necessary to cleanliness, the filtered mordant is allowed to act cold for five minutes, after which it is warmed and then in one minute washed off. After drying, the smear is stained with the carbol-fuchsin or carbol-gentian violet solution, and then washed, dried and mounted. (Plate II, Figs. 18 to 21.)

Frequently the flagella appear well stained, but often the process has to be repeated a number of times. Overheating of the film prevents the staining of the flagella. The cell membrane may also show by this method.

Van Ermengen's Method gives good results. It is as follows: The films are placed for one hour at room temperature, or are heated for five minutes over a water-bath at 100° C. in the following solution: *Solution A*, osmic acid, 2 per cent. solution, 1 part; tannin, 10 to 25 per cent. solution, 2 parts.

Wash successively with water, absolute alcohol and water, then place in the following solution for a few seconds: *Solution B*, 0.5 per cent. solution of $AgNO_3$ in distilled water.

Without washing transfer them to a third solution: *Solution C*, gallic acid, 5 gm.; tannin, 3 gm.; fused potassium acetate, 10 gm.; distilled water, 350 c.c.

After keeping in this for a few seconds, place again in Solution B until film begins to turn black. Then wash and examine.

Kulp's Modification of Löffler's Method.[2]—The bacillus is cultivated for at least two generations on agar slants having an appreciable amount of condensation water. Transfers are made from the condensation water. One or 2 drops of the condensation water from the final culture are poured into a tube of sterile water (15 to 20 c.c.) and incubated forty-eight to seventy-two hours. A loopful of this suspension is transferred to a carefully cleaned slide and dried quickly in the incubator. When dry apply the mordant for fifteen minutes at room temperature. It is then washed carefully and treated with the stain for

[1] W. H. Wright (Stain Technology, 1928, **3**, 14) gives a review of various methods with bibliography.

[2] Kulp, W. L.: Stain Technology, 1926, **1**, 60.

fifteen minutes at room temperature. The excess stain is removed by washing and the slide allowed to dry.

Mordant.

Tannic acid, 20 per cent. aqueous solution	50 c.c.
Ferrous sulphate, saturated aqueous solution	25 "
Basic fuchsin, saturated 95 per cent alcohol solution	5 "

Stain.

Anilin water, saturated, fresly filtered	100 c.c.
Basic fuchsin, saturated 95 per cent alcohol solution	12 "

The preparation of the smear may be modified as follows: Place a comparatively large loop of the suspension at one end of the slide. Hold the slide in a vertical position and allow the drop to run down the slide. This will dry quickly in the incubator. Very satisfactory results have been obtained by this method.

Weiss Method.[1]—*Mordant.*—Solution "A": 100 gm. tannic acid in 100 c.c. 95 per cent alcohol. Solution "B": 7.5 c.c. glacial acetic acid in 100 c.c. formalin (40 per cent formaldehyde). For use mix 1 part of "A" with 2 parts "B".

Stain.—Five grams of gentian or crystal violet are added to 100 c.c. formalin, shaken, heated slightly, allowed to stand one week at room temperature and then filtered.

Procedure.—To an eighteen-hour broth culture add glacial acetic acid (q.s. ad 5 per cent.). Hold at 37° C. for fifteen minutes. Transfer loopful to clean slide and allow to dry. Cover with the mordant, steaming for two to five minutes. Wash slide with warm water for one to two minutes and then cover with stain for one to two minutes. To increase the density of staining of flagella, a further treatment with an acid stain (acid green, 10 per cent in diluted alcohol, 2 parts distilled water and 1 part 95 per cent. alcohol) for ten minutes. The slide is then washed and dried.

Methods of Staining Spirochetes.—MacNeal's and Giemsa's methods give excellent results (pp. 80 and 81).

Silver Impregnation Method.—In Smears.—Until recently the demonstration in smears of the syphilis spirochete by the *silver impregnation method*, so successfully used by Levaditi in section, has been unsatisfactory. Stern, however, and Flexner corroborating him, have gotten beautiful results by the following simple method:

(*a*) Air-dried in 37° incubator for some hours.

(*b*) Ten per cent. aqueous silver nitrate for some hours (Flexner thinks three or four days' exposure better) in diffuse daylight.

(*c*) When the brownish color reaches a certain tone (easily recognized after experience) and when a metallic sheen develops, the slide is washed well in water, dried and mounted.

The blood cells are well preserved, they have a delicate dark brown contour, and contain fine light brown granules. The spirochetes are deep black on a pale brown and in places a colorless background.

Other spirochetal organisms may be silvered by this method, but as they may be differentiated with greater difficulty than with Giemsa's stain, the latter should always be used as well.

These organisms may also be demonstrated by the India-ink method (see p. 77).

In Sections.—Sections are prepared by the *silver impregnation method of Levaditi* as follows.[2] Fix small pieces of tissue 0.5 mm. in thickness for twenty-four to forty-eight hours in formalin, 10 per cent. Wash in 95 per cent. alcohol twelve to sixteen hours. Wash in distilled water until the pieces sink. Impregnate two or three hours at room temperature and four to six hours at 50° C. in the following fluid: Nitrate of silver, 1; pyridin, 10 (add just before using);

[1] Weiss, E.: Jour. Inf. Dis., 1928, **43**, 228.

[2] Levaditi and Manouelian: Compt. rend. Soc. de biol., 1906, **60**, 134.

distilled water, 100. Wash rapidly in 10 per cent. pyridin. Reduce the silver by placing in the following mixture for several hours: Pyrogallic acid, 4; acetone, 10 (add just before using); pyridin, 15; distilled water, 100. Harden in alcohol, xylol, paraffin. Levaditi's first method is longer but more reliable. Fix small pieces in formalin, 10 per cent. Harden in 95 per cent. alcohol. Wash in distilled water several minutes. Impregnate three to five days at 37° C. in 1.5 per cent. solution silver nitrate. Reduce twenty-four hours in: Pyrogallic acid, 4; formalin, 5; water, 100. Imbed in paraffin. By these methods the spirochetes appear densely black.

Warthin-Starry[1] *Silver Agar Method of Staining Spirochetes in Cover-glass Films.*—(1) Prepare smears on No. 1 (*perfectly clean*) cover-glasses. (2) Dry *thoroughly* in air. (3) Place in absolute alcohol three to five minutes. (4) Wash in distilled water. (If hydrogen peroxide is used to clear background, the smear is placed in concentrated hydrogen peroxide for five to twenty minutes then washed thoroughly in distilled water). (5) Rinse cover-glass with smear in 2 per cent. silver nitrate. Cover smear side with another perfectly clean cover-glass also rinsed in the silver nitrate solution. Place the adherent pair of cover-glasses carefully, so as not to separate them, in 2 per cent silver nitrate, and place in incubator for one to two hours; then remove the cover-glasses from the silver nitrate solution and separate them. (6) Place cover-glass with smear side up in following mixture: 2 per cent. silver nitrate solution, 3 c.c.; warm 10 per cent. aqueous gelatin solution, 5 c.c.; warm glycerol, 5 c.c.; warm 1.5 per cent. agar suspension, 5 c.c.; 5 per cent. aqueous hydroquinone solution, 2 c.c. (7) After the solution is reduced remove and rinse in 5 per cent. sodium thiosulphate solution. (8) Rinse in distilled water. (9) Absolute alcohol, xylol, balsam.

Ross's Method of Examining a Large Quantity of Malarial Blood in One Film.—A large drop of blood (about 20 c.mm.) is placed on a glass slide and is slightly spread over an area which can be covered by an ordinary cover-glass. This is allowed to dry in the air or it is warmed over a flame without heating it more than enough to fix the hemoglobin. The dry film is first treated with a solution of acetic acid, washed in water and then covered with an aqueous solution of eosin (10 per cent.) and allowed to remain about fifteen minutes. This is then gently washed off and a weak alkaline methylene-blue solution is run over the film and left for a few seconds, when the preparation is again gently washed. After drying, it is ready for examination.

Staining Method for Negri Bodies (*Williams's*[2] *Modification of Van Giesen's Method*).—Smears partially air-dried are fixed for ten seconds in neutral methyl alcohol to which 0.1 per cent. picric acid has been added. Excess of fixative removed by filter paper. Smears then stained in following solution: Saturated alcoholic solution fuchsin, 0.5 c.c.; saturated alcoholic solution methylene blue, 10 c.c.; distilled water, 30 c.c. The stain is poured on the smear and held over the flame until it steams. The smear is then washed in tap-water and blotted with fine filter paper. The Negri bodies are magenta with blue granules, the nerve cells blue, and the red blood cells yellow, or salmon color (Plate X, Fig. 1). This staining mixture may be kept in the ice-box for a long time.

Heidenhain's Iron-hematoxylin Stain.—(*a*) Mordant and differentiating fluid: Iron oxyammonium sulphate, 2.5 grams; distilled water, 100 c.c. (*b*) Staining fluid: Hematoxylin, 1 gram; alcohol, 10 c.c.; distilled water, 90 c.c. (To be kept in a red bottle and allowed to stand for about four weeks before using.) This method is very good for staining yeasts and protozoa (see Chapter on Amœbæ) in tissues.

Preservation of Smears.—Dry stained preparations of bacteria keep indefinitely, but if mounted in Canada balsam, cedar oil, or dammar lac they tend gradually to fade, although many preparations may be

[1] Jour. Infect. Dis., 1922, **30**, 592.
[2] Am. Jour. Pub. Hyg., 1908, **18**, No. 1.

preserved for many months or years if the preservative is neutral. Dry unstained spreads should be kept in the ice-box until stained.

Examination of Microörganisms in Tissues.—Occasionally it is of importance to examine the organisms as they occur in the tissues themselves. The tissues should be obtained soon after death, so as to prevent as much as possible postmortem changes, with consequent increase or decrease in the number of microbes. Selected pieces of tissues can be frozen by ether or carbon dioxide and sections cut, but the best results are obtained when the material is imbedded in paraffin or in celloidin.

Fixing and Hardening Tissues.—From properly selected portions small pieces, not larger than $\frac{1}{4}$ inch by $\frac{1}{8}$ inch, are removed and placed in one of the following fixatives arranged in order of their comparative worth in demonstrating microörganisms.

1. *Zenker's Fluid.*—Add to a solution of Müller (potassium bichromate, 2 to $2\frac{1}{2}$ parts; sodium sulphate, 1 part; water, 100 parts) 5 per cent. of saturated sublimate solution and, when ready to use, 5 per cent. of glacial acetic acid. Moist spreads are fixed for one to five minutes, small pieces of tissue for three to twelve hours. They are then washed with water or put immediately into successive alcohols.

2. *Corrosive Sublimate.*—Corrosive sublimate (saturated solution in 0.75 per cent. sodium chloride solution) is an excellent fixing agent. Dissolve the sublimate in the salt solution by heat, allow it to cool; the separation of crystals will show that saturation is complete. For pieces of tissue $\frac{1}{8}$ inch in thickness four hours' immersion is sufficient; for larger, twenty-four hours may be necessary. They should then be placed in pieces of gauze and left in running water for from twelve to twenty-four hours, according to the size of the pieces, to wash out the excess of sublimate.

3. *Sublimate Alcohol.*—Hot sublimate (saturated) alcohol (50° C.) or saturated sublimate, to which 5 per cent. glacial acetic acid may be added. The preparation should remain in it a few seconds, then should be washed for one-half hour in 60 per cent. iodine-alcohol, and then placed in 70 per cent. alcohol. They may remain here for an indefinite time, until they are to be stained, when they are rinsed in distilled water and then placed in the staining fluid.

4. *Absolute Alcohol.*—Absolute alcohol for from four to eight hours, and longer if thicker. For the larger pieces it is better to change the alcohol after eight hours. The pieces of tissue should be kept from falling to the bottom, as the higher layers of alcohol remain nearer absolute. If along with the microorganisms one wishes to study the finer structure of the tissue, it is better to employ one of the other fixatives.

5. *Osmic Acid.*—Two per cent. osmic acid (to be kept in a red glass vial with a ground-glass stopper). Moist smears are exposed to its fumes for a few seconds, small pieces for sections, four to eight hours, then carried through the various alcohols and xylol and mounted or imbedded in the usual way.

6. *Hermann's Fluid.*—A 1 per cent. solution platinum chloride, 15 c.c.; a 2 per cent. solution osmic acid, 4 c.c.; glacial acetic acid, 1 c.c. Moist spreads may be fixed for several minutes; very small pieces of tissue for twenty-four hours.

7. *Formalin.*—For fixing in formalin the tissue is put in 4 to 10 per cent. formalin solution (the 40 per cent. commercial solution) for three to twenty-four hours, and then in successive strengths of alcohol.

To *harden* they are placed successively for twenty-four hours each in the following strengths of ethyl alcohol: 30 per cent., 69 per cent. and 90 per cent. Finally they are placed in absolute alcohol for twenty-four hours which dehydrates them and they are then ready to be imbedded in paraffin.

To imbed in paraffin, the pieces are put in: (1) Cedar oil until translucent; (2) cedar oil and paraffin, equal parts, at 37° C. for two hours; (3) paraffin 52° C. two hours in each of two baths. They are then boxed ready for sections.

Sections are cut at 3 to 6μ, and are dried at 36° C. for about twenty-four hours, protected from dust. Xylol may be used instead of cedar oil.

The paraffin sections of tissue having been prepared and cut, they are ready for staining after the paraffin is removed. If all of the sublimate has not been removed by the water, the sections may be immersed in iodine-alcohol for ten minutes.

Löffler's Staining Method for Sections.—The section is placed in Löffler's alkaline methylene-blue solution for five to thirty minutes, decolorized for a few seconds in 1 per cent. acetic acid. It is then placed in absolute alcohol, xylol and Canada balsam. The number of seconds during which the preparation remains in the acetic acid must be tested by trials.

Stain for Influenza Bacilli in Tissues.—MacCallum[1] recommends a combination of Goodpasture's and Weigert's stains as follows:

Fixation should be in Zenker formaldehyde solution, and very thin paraffin sections should be used.

1. Stain for from ten minutes to one-half hour or more in Goodpasture's stain, which is:

	Gm. or c.c.
30 per cent. alcohol	100.00
Basic fuchsin	0.59
Anilin	1.00
Phenol crystals	1.00

2. Wash in water.
3. Differentiate in 40 per cent. formaldehyde solution. This requires only a few seconds. The bright red color washes away and gives place to a clear rose.
4. Wash.
5. Counterstain in saturated aqueous picric acid. The section remains until it assumes a purplish yellow, about three to five minutes or less.
6. Wash in water.
7. Differentiate in 95 per cent. alcohol. The red reappears and some of it is washed out. Some of the yellow of the picric acid is also washed out.
8. Wash in water.
9. Stain in Stirling's gentian violet five minutes or more.
10. Wash in water.
11. Gram's iodin solution.
12. Blot dry without washing.
13. Anilin and xylene (equal parts) until no more color comes away.
14. Two changes of xylene.
15. Balsam.

Gram-negative organisms stain red, gram-positive blue. The tissues stain brilliantly in shades of red and purple. Fibrin is, of course, sharply stained, and the granules of mast cells are prominent as red or purplish coccus-like bodies.

The method should be useful in the study of infections of exposed surfaces, such as the intestinal mucosa and the urinary bladder, with gram negative organisms. The lesions of dysentery, typhoid fever and many other similar conditions might be studied with advantage by this method.

Methods of Examination of Ringworm Fungus.—*Living Specimens.*—Place scales or hairs in warm liq. potassæ (20 per cent.) for a few seconds. Examine under cover-glass.

Permanent Specimens.—Remove fat with chloroform, then place material in formic acid and heat to boiling (two or three minutes). Remove acid by washing in distilled water, stain with Löffler's methylene blue. Wash, dehydrate in absolute alcohol, clear in xylol and mount in balsam.

[1] Jour. Am. Med. Assn., 1919, **72**, 193.

CHAPTER IV.

GENERAL METHODS USED IN THE CULTIVATION OF MICROÖRGANISMS.

GLASSWARE, CULTURE MEDIA, STERILIZATION, METHODS OF CULTIVATION AND ISOLATION, CULTURAL CHARACTERISTICS.

The methods employed for the artificial cultivation of microörganisms are of fundamental importance. By their use we can obtain one variety growing apart from all others, namely, in pure culture. This pure culture may be planted on various media and the morphological, biochemical, and cultural characteristics studied for classification and identification. It is evident that all glassware and instruments used must be free from other microörganisms; that is, they must be sterile.

GLASSWARE.

Various types of glassware are needed: such as test tubes, flasks, bottles, petri dishes (often called "plates"), pipettes, etc. (Figs. 15 to 21). Before glassware is subjected to the necessary sterilization, noted above, it should be chemically clean.

Preparation of Glassware.

New glassware requires not only a thorough mechanical cleansing with soap and hot water and the loosening of adherent dirt by means of test-tube or bottle brushes, but also special attention to render it free from the *resistant spores* which are often present in the straw or other packing material. Such spores resist frequently the high temperatures of hot-air sterilization (p. 94), but they may be destroyed by boiling the glassware in a strong soap solution (5 per cent.) for one hour. Because many of the soap powders cloud glassware they are to be avoided; instead add to the water thin shavings of a good brown soap.

Old glassware containing cultures, especially those of tetanus and anthrax, should be sterilized in the autoclave at 15 pounds' pressure for one-half hour; less resistant cultures may be killed by boiling for one hour in a covered boiler, containing a 5 per cent. soap solution which should cover and fill the glassware from which all stoppers have been removed. The cotton stoppers should be disinfected by burning or other means. Any solid medium present is melted and the glassware then washed according to the directions given above.

The *rinsing* must be thoroughly done in running water to remove the soap. The glassware should then be allowed to drain and dry before being plugged.

Neutralization of Glassware.—Where slight changes of reaction are of importance the glassware should be neutralized after washing. Soak in 1 per cent. hydrochloric acid for several hours or boil for one-half hour, wash free of acid, rinse in tap water then in distilled water. New glassware, especially of the cheaper grades, is most likely to give off free alkali.

Plugging.—All narrow-mouthed containers are plugged in such a way as to exclude dust and germs. Use ordinary non-absorbent cotton (cotton batting).

The cotton should not be twisted in, as creases will form along the sides of the glass, leaving channels for contamination. Either fold the cotton into a plug

FIG. 15.—Test-tubes for small amounts of media and individual cultures; if the medium be agar, it is either slanted while liquid or used in 10-c.c. amounts to be melted and poured into Petri dishes. Tubes without lip can be also used. Average size, 6 x $\frac{5}{8}$ inches.

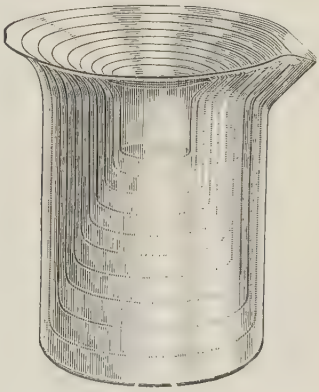


FIG. 16.—Nest of beakers.

or take a square, fold one corner and place a rod against the fold to push the cotton into the neck of the container or tube. A sufficient amount of cotton should project for handling and the plug should be just tight enough to allow one

Globe. Volumetric. Erlenmeyer.

FIG. 17.—Flasks for preparing culture media (collecting filtrates) or for growing bacteria in liquid media, as for production of diphtheria and tetanus toxin. Capacity 500 to 2000 c.c.

to lift the container by means of the plug. Several thicknesses of filter paper may be used to cover beakers and other wide-mouthed containers.

Sterilization.—After the glassware is plugged it is sterilized by dry heat. Any type of hot-air sterilizer may be used (Fig. 22). Heat at a temperature

FIG. 18.—Type of bottle commonly used for dilutions, etc., or as substitute for small flasks, capacity 100 c.c.

FIG. 19.—Blake bottle. Laid on its flat side it gives a large surface of broth or agar. For preparation of vaccines and tuberculin, capacity, 1 quart and 1 pint.

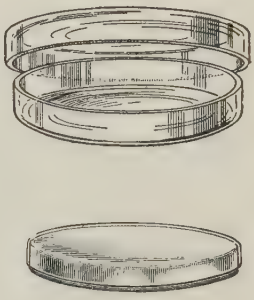
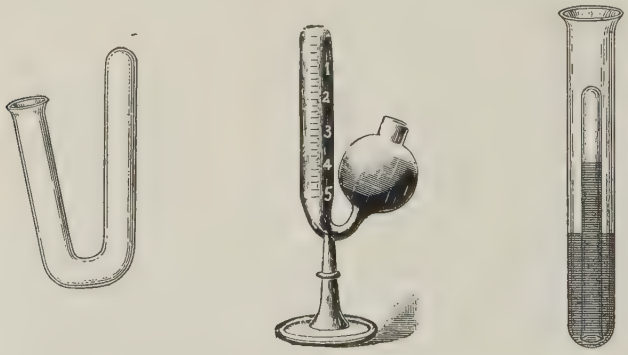


FIG. 20.—Petri dishes. Commonest size is 10 cm. in diameter. For solid media only, chiefly for mixed cultures, either surface growth or scattered through medium (agar or gelatin) while in liquid state; medium then hardens in plate. Individual colonies, growing separately after incubation, can be fished and transferred to separate tubes.

of 160° to 170° C. for one hour. This heating not only sterilizes the plugged glassware but sets the shape of the cotton plugs. Keep the sterilizer closed when the sterilization is finished so that the cooling may be gradual, otherwise the glassware may crack. When large amounts of glassware must be handled, the



a *b* *c*

FIG. 21.—Types of fermentation tubes for liquid media to show production of gas by inoculated organisms. The gas collects in closed portion of tube displacing liquid.

use of a good thermo-regulating valve on the sterilizer together with a recording thermometer, gives excellent results and releases the attendant for other work.

CULTURE MEDIA.

General Considerations.

Most microörganisms causing disease require complex foodstuffs (see p. 53) of suitable reaction (see Reaction) and similar in constitution to those in the animal body. The general basis of media for these types is an *infusion* or *watery extract of meat* which contains soluble albumins, extractives, salts, carbohydrates and coloring matter. In many respects

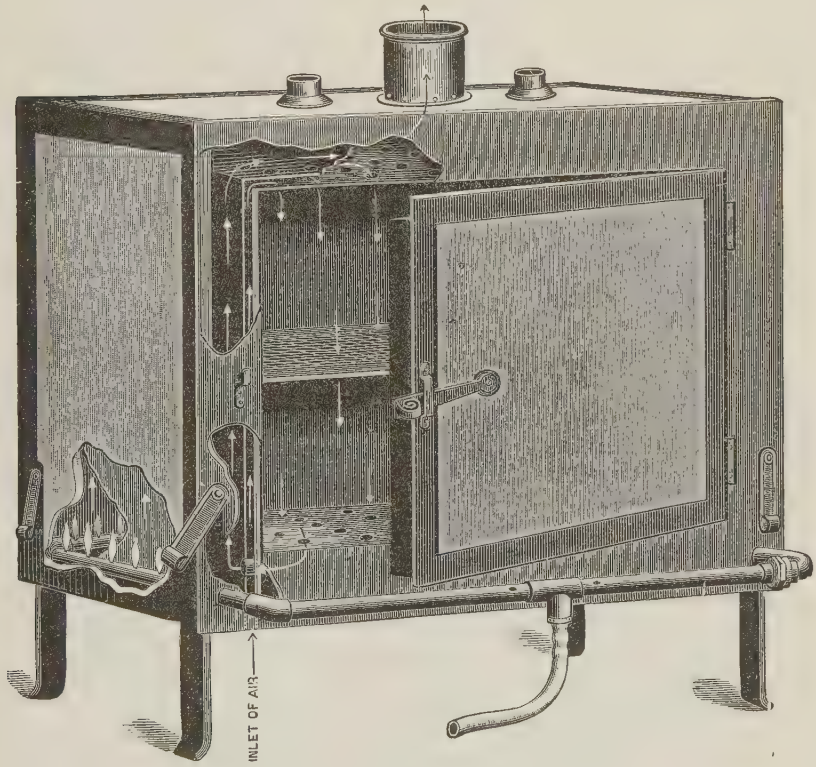


FIG. 22.—Hot-air sterilizer. Lautenschläger form.

this infusion of meat would make a good medium, but the application of heat necessary for its sterilization affects some of its constituents deleteriously by changing their composition, thus causing their loss as foodstuffs. After such changes a very clear medium may be obtained by filtration but the lost nutritive material must be replaced in part, at least, by the addition of some other substance. *Peptone*, which is soluble in water and is not precipitated by boiling, fills this need to quite an extent.

As an alternative basis for the above infusion of meat, potted *beef extract* (Liebig's, Armour's, etc.) may be used. It contains extractives or meat flavor and mineral salts but the prolonged heating in the method of manufacture causes a great loss of its nutritive elements. The addition of peptone restores these in part only. Beef extract is, however, very useful for certain kinds of media and is of great convenience in small laboratories or those in tropical regions.

To meet the needs of some of the pathogenic organisms other nutritive substances, such as serum or blood, are frequently added to the sterilized medium. Carbohydrates may also be added. On the other hand, the non-pathogenic organisms vary in their ability to flourish on these more complex media, for some will grow only in simple media containing inorganic salts.

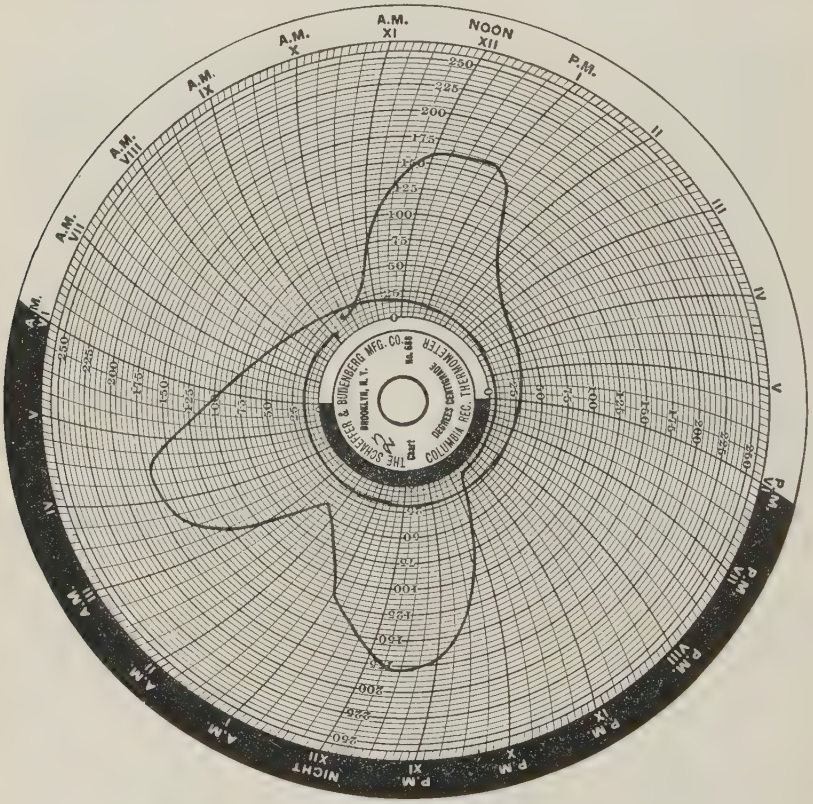


Fig. 23.—Recording chart of hot-air sterilizer.

The media outlined above are fluid in character but their consistency may be modified by the addition of suitable amounts of gelatin, agar or albumin. The first two solidify when cooled and the third is coagulated by heating; thus the media become either *solid* or *semisolid* as desired. *Gelatin* gives a very clear transparent solid medium but as its melting point (20° C.) is below that required for the growth of patho-

genic organisms (37° C.) its usefulness is limited. *Agar*,[1] a gel of vegetable origin, dissolves in water at the boiling-point (100° C.) and remains liquid until cooled down to about 40° C. Most media containing agar are quite clear if properly made. *Coagulated albumin*, as typified in

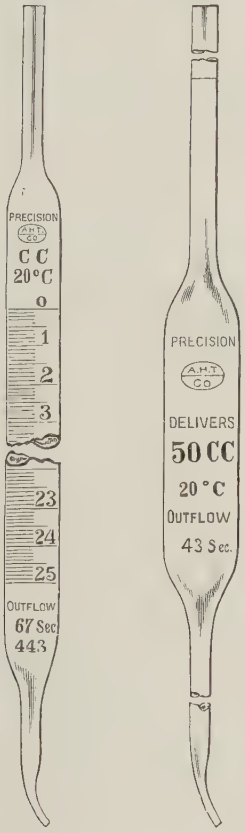


Fig. 24

Graduated pipette. Volumetric pipette.

Obtainable in various sizes and graduations for measuring accurately definite amounts of fluids in preparation of dilutions, etc.

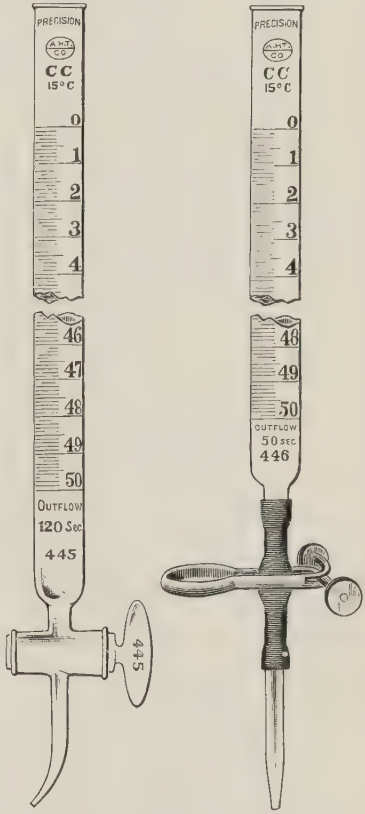


Fig. 25

Burettes. Most convenient type has a blue line against a white background on back, giving sharper readings; to be held in clamp on ring-stand; used in titration.

[1] The ordinary market agar is a variable product. Although it is "graded" commercially there is no way of telling whether or not a grade is suitable for bacteriological purposes unless it is tested. The solidifying quality depends, as with fruit jellies, on the pectin content. It is advisable, therefore, to test a sample of each lot ordered for this substance. The pectin should be present to the extent of at least 90 per cent.

Test for Pectin.—Wash 8 to 10 grams of thread agar rather rapidly in cold water. Dry at 37° to 50° C. overnight. Weigh out 5 grams and dissolve them in 1000 c.c. of distilled water to which has been added 2 per cent. of sodium chloride. The dissolving of the agar may be done by boiling on the stove or by heating in the autoclave or Arnold. Add 2 liters of alcohol (95 per cent.) to precipitate the pectin. Let stand overnight. Dry a filter paper overnight at 37° C. and then weigh it. Filter the mixture through the weighed paper. Dry the residue on the paper at 37° C. Weigh again, calculate the amount of residue and then calculate the percentage.

Löffler's blood serum, provides both firmness and a certain amount of food, but such a medium has the drawback of being opaque.

Solid media were first designed for keeping colonies of organisms separate so they could be fished and pure cultures obtained. Such media have been found excellent for other purposes generally and are more used than fluid media.

Semisolid media, prepared either with agar in lessened amounts or agar and gelatin mixed, are especially useful for keeping routine stock cultures alive for long periods of time without transplanting.

Method of Preparing Culture Media.

The meat used most generally is either beef or veal. It must be carefully freed from fat, fascia, etc. This is done preferably at the laboratory just before the meat is chopped fine or put through the meat grinder. Chopped meat bought from the butcher, as such, is not desirable, as it contains too much fat. In ordering meat allowance should be made for the waste of fat and bone. Beef hearts, if obtained fresh, are economical and have been found satisfactory for various purposes.

Nutrient Broth.

Chopped meat (veal or beef), 5 pounds.[1]
Tap water, 5000 c.c.
Soak overnight at ice-box or at room temperature.

Method I.

Strain through cheese-cloth; squeeze by twisting cloth or use meat press. Discard raw meat. Fluid = *meat juice.*

Method II.

Weigh kettle and contents. Heat at 45° C. for one hour, then boil half hour. Make up by weight loss due to evaporation by adding hot water. Strain through cheese-cloth. Squeeze by twisting cloth or use meat-press. Discard cooked meat. Fluid = *meat infusion,* which may be sterilized in flasks and stored for future use.

Add: Peptone, 1.0 per cent.
Sodium chloride, 0.5 per cent.

Heat to 50° C. to dissolve peptone and salt. Determine reaction[2] (see Reaction).

Heat to boiling. Determine reaction[3] (see Reaction).

Set reaction by adding the calculated amount of normal sodium hydroxide. Boil or autoclave for ten minutes to throw down precipitate.

Determine reaction[3] again and adjust if necessary. (In this case boil again). Filter through cotton and filter paper in glass funnel.

Sterilize in tubes or flasks by heating in autoclave for one-half hour at 15 pounds' pressure.

[1] These directions are for an amount larger than the 1 liter often suggested, because the proportionate loss by evaporation is much less.

[2] If phenolphthalein be used as the indicator, boil the test sample of broth in casserole to approximate roughly future reaction of kettleful of medium. This is the only point where the *boiling* titration is the correct procedure. (See p. 106).

[3] If phenolphthalein be the indicator, use room temperature method of titration.

Explanation of Methods.—In Method I less heat is used with consequently less change of nutritive material. The whole process of making the broth is carried out at one time. It is, however, somewhat wasteful in that the raw meat still containing some nutriment is discarded. On the other hand the broth is particularly suitable for some special purposes—such as the preparation of pneumococcus vaccine (pneumococcus broth) or the growth of other delicate organisms.

When large amounts of media are prepared by this method the raw meat may be reinfused, using only 1 part of water to 2 or 3 of meat, and proceeding as in Method II. This "residue infusion" forms a suitable base of agar media for the less exacting microörganisms.

Double strength broth may be prepared by using 2 pounds of meat to the liter.

With Method II the greater amount of heating causes greater loss of foodstuffs especially if the infusion is sterilized for storing with the reaction unadjusted. The advantages, however, of having on hand stock meat infusion ready for further preparation in various ways, outweigh the objections, since media made from such stock give excellent results in general work.

The following *variant* is sometimes useful in place of nutrient broth:

Beef Extract Broth.—Beef extract (Liebig's or Armour's, etc.), 2 to 5 grams; water, 1000 c.c.; peptone (1 per cent.), 10 grams; sodium chloride (0.5 per cent.), 5 grams.

Heat to boiling over fire to dissolve ingredients. Boil fifteen minutes. Determine reaction (see Reaction) and adjust if necessary by addition of normal sodium hydroxide. Boil ten minutes to throw down precipitate. Filter through cotton and paper in glass funnel. Put in tubes or flasks and sterilize in autoclave one-half hour at fifteen pounds' pressure.

Reaction.—General considerations. The meat bases for culture media and also peptones are naturally more or less acid in comparison with true neutrality or distilled water (page 101). Most pathogenic organisms grow best in media with a reaction near true neutrality or slightly alkaline to it, therefore it is necessary to adjust the reaction of media to the most suitable point by the addition of an alkali such as sodium hydroxide or sodium carbonate.

The oldest methods were very simple—litmus or litmus paper (p. 104) which changes color at about true neutrality was used as an indicator (p. 103) and the acidity reduced to neutral or to slightly alkaline by adding a 4 per cent. solution of sodium hydroxide. Later phenolphthalein was introduced together with the *titrimetric method* (p. 105) and media were made slightly acid to this indicator. The errors of this method are pointed out on page 106. The *electrometric* and *colorimetric* methods (p. 102) recently introduced for obtaining the hydrogen-ion concentration or actual acidity (p. 101) of culture media give much more accurate results.

Determination of Reaction.[1]—The determination of the true reaction, *i. e.*, actual acidity, requires an understanding of the theory of hydrogen ions. This

[1] This explanation is based chiefly on two articles (1) A. L. Wachter: Personal Communication. (2) H. J. Conn, Chairman: Methods of Pure Culture Study. Report of Committee on the Chart for Identification of Bacterial Species, Jour. Bacteriol.. March, 1919, **4**, 2, 107.

theory of electrolytic dissociation was brought forward in 1887 by Arrhenius. When a substance of a certain class (acid, salt or base) goes into solution in water some of it remains in the form of molecules while other molecules are split into two parts—positive ions carrying a positive charge and negative ions carrying a negative charge of electricity. *Example.*—Normal hydrochloric acid (HCl), which is a solution of the acid in water, contains molecules of HCl to the extent of 22 per cent., while the dissociated portion is made up of positive hydrogen ions (H-ions) and negative chlorine ions (Cl-ions) which together form 78 per cent. of the whole.

A substance in the state of ions is entirely different from the molecular substance; for example, dry hydrogen (molecular) is tasteless and not acid. On the other hand, hydrogen *ions* are acid and give a watery solution containing them its sour taste and other acid properties. If their electrical charge is removed by withdrawing the solvent water, two H-ions unite to become a molecule of hydrogen (H) and lose their acid properties.

In an aqueous solution of sugar the molecules are not dissociated to any extent, if at all. Solutions of this character do not conduct electricity.

Pure water (H_2O) contains both positive ions (H) and negative ions (OH) in equal amounts which neutralize each other. $\overset{+}{H} + \overset{-}{OH}$ If to pure water we add a drop of HCl we have a mixture containing H-ions, OH-ions and Cl-ions. The number of free H-ions is greatly increased because of the large number in the drop of HCl. The number of OH-ions is decreased because the change in ionic equilibrium caused many to combine with free H-ions to form water. If a larger amount of HCl is added the H-ion concentration increases and the OH-ion concentration decreases. If a *base* is added to water or other neutral solution the relation is exactly the reverse—the concentration of OH-ions is increased and the concentration of H-ions decreased. Either the H-ion concentration or the OH-ion concentration may be used as an *index* (p. 103) but it is customary to use the H-ion concentration which is inversely proportional to the dissociation of the base and therefore to the true alkaline reaction of the solution.

The degree of dissociation or ionization of different substances in solution in water varies. In dilute solutions nearly all of the molecules are dissociated; in stronger solutions the molecules are less dissociated. Acids and bases dissociate or ionize to a greater extent than water and in turn the strong inorganic acids, as nitric and hydrochloric, ionize to a greater extent than the weaker acids such as acetic and other organic acids. The H-ion content or concentration of a solution increases with dissociation or ionization; therefore, if different acids are dissolved in the same ratio in water, the H-ion concentration (CH) is an index of the strength of each acid. Examples—normal (N/1) acetic and decinormal (N/10) hydrochloric acids in chart (p. 107).

Normal Acid.—The older definition reads "one containing in 1 liter as much acid as contains 1 gram-atom of replaceable hydrogen." The newer definition is as follows: "A solution containing sufficient acid to furnish 1 gram of ionizable (*i. e.*, capable of dissociation) hydrogen per liter." All the hydrogen, however, may not be dissociated at one time. We have seen (p. 100) that in a normal solution of HCl only about 78 per cent. (or 0.78 of a gram) of the hydrogen is dissociated or in the form of ions at 20° C. The remaining 22 per cent. (0.22 gram) is combined with chlorine as molecules of HCl. In *decinormal* HCl, at 20° C., about 92 per cent. of the 0.1 gram of hydrogen is dissociated or in the form of ions. In higher dilutions the dissociation or ionization of the amount of hydrogen present is almost complete.

Normal Alkali.—Just as a liter of N/1 acid contains 1 gram of ionizable hydrogen so a liter of N/1 alkali contains an amount of ionizable hydroxyl chemically equivalent to the 1 gram of hydrogen; for example, normal sodium hydroxide (NaOH) contains 17 grams of OH (atomic weight of O = 16, of H = 1).

The gradations of H-ion concentrations fall for the most part in a reaction range of special interest to the biochemist, the physician and the bacteriologist. This range includes the reactions of normal solutions and their dilutions, the blood and their body fluids, vegetable and fruit juices, culture media, etc., and covers the region in which enzymes are active.

In 1909, *Sörensen*[1] published a *scale* for expressing the reactions in this range. Such a scale is of great value for the following reasons:

A normal acid contains as we have seen, 1 gram of hydrogen in a liter of solution; consequently any dilution of the normal solution contains a fraction of the gram of hydrogen. As the dilution is increased, the amount of hydrogen decreases but the figure of the fraction becomes larger and larger. So also in the blood and other biochemical fluids, the actual amounts of hydrogen present are very small and the figures expressing them are very large. Even though these fractions, for example, $\frac{1}{10000}$ (or .00001) be written in the form 1 x 10^{-5} (minus exponent because of the decimal) the expressions are unwieldy. Sörensen suggested using the exponent only, together with the designation pH;[2] thus, 1×10^{-5} = pH 5.

There is only $\frac{1}{10000000}$ of a gram of hydrogen in a liter of distilled water. This fraction = 0.0000001 or 1 x 10^{-7} or $\log^{-7}$ and, according to Sörensen, is pH 7. Distilled water represents true neutrality because the positive (H) ions and the negative (OH) ions are present in balanced amounts and as fast as dissociation occurs the H-ions and OH-ions recombine to form H_2O; H-ion + OH-ion = H_2O. A solution containing an amount of H-ions greater than $\frac{1}{10000000}$ of a gram per liter is acid; one containing less than $\frac{1}{10000000}$ gram per liter is alkaline. Acid solutions, therefore, have a pH value of less than 7 and alkaline solutions a pH value greater than 7.

In *Sörensen's scale* these figures are arranged from pH 0 to pH 14, pH 0 representing the amount of ionizable hydrogen in a normal solution, for in this case CH = 1, and since the log of 1 = 0, its pH is 0. The relations of pH values to acid and alkali normalities and H-ion concentrations have been arranged in a table by Wachter (p. 103). In this table absolute accuracy[3] as a whole has been sacrificed for simplicity of presentation.

The zero of Sörensen's scale may be considered as a plus or minus (+0) and the scale thus linked to those dilutions of acid which are stronger than normal solutions.

The amount of dissociation occurring in any solution is affected by the degree of dilution, temperature, presence of other substances known as *buffers*, and also other factors. A *buffer* is any substance (present in a solution) which because of its affinity for either acid or alkali tends to preserve the original H-ion concentration of the solution by combining with any acid or alkali which may be added thus nullifying their effect. Peptone, meat infusion, sodium borate, dipotassium phosphate, etc., act as buffers.

The *actual acidity* of a medium depends upon the amount of ionized hydrogen present, *i. e.*, on the H-ion concentration under the existing conditions of temperature, buffers, etc., in other words on the amount of dissociated acid (or alkali) present at a given time. The *total acidity* (or alkalinity) takes into account all the acid (or alkali) present whether dissociated or undissociated. For example, we have seen that a liter of N/1 HCl contains 1 gram of ionizable hydrogen but that only 78 per cent. of this hydrogen is actually ionized. If now a little N/1 alkali is added, some of the dissociated acid is neutralized and at once some of the previously undissociated acid becomes ionized or dissociated to take the place of that portion neutralized. Further additions of the alkali would continue the process until no undissociated acid remained and the H-ions of the dissociated acid would be neutralized. This process when performed

[1] Compt. Rend. Lab., Carlsberg, 1909, **8**, 1.
[2] Sörenson: Compt. Rend. Lab. Carlsberg, 1919, **8**, 1.
[3] We have seen (p. 100) that with N/1 HCl not all of the acid is actually dissociated.

with measured amount of acid and alkali in the presence of an indicator (p. 103), is called *titration* (p. 105).

The usefulness of a medium depends upon its actual acidity and whether this is suitable for the particular organisms which are to be cultivated. This actual acidity, or H-ion concentration, may be determined in either of two ways: (1) *electrometrically*, (2) *colorimetrically*. The first is an accurate method and one used chiefly in careful determinations in physical chemistry. The second is based on the first and although it is not quite so accurate, the error is but slight. Its comparatively easy application makes it generally useful especially for bacteriological work.

Electrometric Method.—The medium to be tested is made to act the part of the solution in an improvised cell. By means of special electrodes the slight current (or difference of potential between the electrodes) derived from the electrically charged ions in the medium, is measured against the known current of a standard cell—by means of a potentiometer. Then with the aid of certain mathematical formulæ, the H-ion concentration is calculated. This method is too complicated and difficult for ordinary purposes. To apply this method correctly requires not only complicated electrical apparatus but special training.[1]

Colorimetric Method.—It has been found that certain shades of color are obtained when certain indicators (p. 103) are added each to a specially prepared solution of known H-ion concentration as determined by the electrometric test. Such solutions are known as standard solutions (see below). If a suitable one of the indicators be added to a medium or a solution, whose H-ion concentration we wish to determine, a similar shade of color appears. By deciding which shade of color in the standard set of solutions the shade of the medium (plus the indicator) approaches most nearly, we can determine quite closely its H-ion concentration. Thus the *actual acidity* is determined by a direct reading (or matching of colors).

The color of the culture medium and in some instances its turbidity are the chief sources of error.[2] This changing or masking of the indicator color may be obviated in two ways: (1) It must be remembered that all successful media are buffer solutions; that is, they contain salts that require relatively large changes in acid or base to produce small changes in reaction. The hydrogen-ion concentration of these solutions is not changed materially by the addition of water. It is possible therefore to dilute the color of the medium itself without affecting the reaction. (2) The color of the medium may be superimposed upon that of the indicator by the comparator method introduced by Walpole. (See Comparator Block, pp. 108 and 109).

Standard Solutions—The standard solutions used in the colorimetric method of determining H-ion concentrations are buffer solutions consisting generally of mixtures of some acid and its alkali salt. (Clark and Lubs). These mixtures have definite H-ion concentrations as determined by the electrometric test. They may be prepared either as suggested by Clark and Lubs[3] or after the method of Sörensen. While the range of the mixtures (Sörensen) given below is somewhat limited it will be found useful for many culture media.

Sörensen's Standard Phosphate Solutions.—Make a M/15 solution of each salt using ammonia-free distilled water:

Na_2HPO_4 [4] (anhydrous) 9.47 grams per liter.

KH_2PO_4, 9.08 grams per liter.

[1] Clark, W. M.: The Determination of Hydrogen Ions, 3d ed., 1928.

[2] O. T. Avery: Personal Communication.

[3] Jour. of Bacteriology, 1917, 2, 1.

[4] Ordinary "sodium phosphate" containing an unknown amount of water of crystallization must not be used. This salt and also the KH_2PO_4 especially prepared for this purpose can be obtained from a reliable drug house. Ask for Sörensen's potassium phosphate and sodium phosphate. (Merck's Blue Label).

Prepare mixtures[1] as follows using the greatest accuracy in pipetting:

M/15 Na_2HPO_4. c.c.		M/15 KH_2PO_4. c.c.		H-ion concentration expressed in pH value.[2]
97.5	+	2.5	=	pH 8.3
95.0	+	5.0	=	pH 8.0
92.0	+	8.0	=	pH 7.8
88.0	+	12.0	=	pH 7.6
82.0	+	18.0	=	pH 7.4
73.0	+	27.0	=	pH 7.2
62.0	+	38.0	=	pH 7.0
50.0	+	50.0	=	pH 6.8
37.0	+	63.0	=	pH 6.6
26.0	+	74.0	=	pH 6.4
18.0	+	82.0	=	pH 6.2
12.0	+	88.0	=	pH 6.0
8.0	+	92.0	=	pH 5.8

A set of tubes which is more easily prepared and which gives roughly the same shades of color (when an indicator is added), as the Standard set, is suggested by Medalia,[3] to whom the student is referred for details.

HYDROGEN-ION CONCENTRATION.[4]

	pH values.	Acid and alkali normalities.	Ch values[6] or grams per liter of hydrogen ion
Acid:	pH 0.0	$\frac{N}{1}$	1
	pH 1.0	$\frac{N}{10}$	$\frac{1}{10}$
	pH 2.0	$\frac{N}{100}$	$\frac{1}{100}$
	pH 3.0	$\frac{N}{1000}$	$\frac{1}{1000}$
	pH 4.0	$\frac{N}{10000}$	$\frac{1}{10000}$
	pH 4.5	Color change—methyl orange	$\frac{3.2}{100000}$
	pH 5.0	$\frac{N}{100000}$	$\frac{1}{100000}$
	pH 6.0	$\frac{N}{1000000}$	$\frac{1}{1000000}$
	pH 6.8	Color change—litmus	$\frac{1.6}{10000000}$
Neutral:	pH 7.0	$\frac{N}{10000000}$	$\frac{1}{10000000}$
	pH 7.5	Blood	$\frac{3.2}{100000000}$
	pH 8.0	$\frac{N}{10000000}$	$\frac{1}{100000000}$
	pH 8.5	Color change—phenolphthalein[7]	$\frac{3.2}{1000000000}$
Alkaline:	pH 9.0	$\frac{N}{100000}$	$\frac{1}{1000000000}$
	pH 10.0	$\frac{N}{10000}$	$\frac{1}{10000000000}$
	pH 11.0	$\frac{N}{1000}$	$\frac{1}{100000000000}$
	pH 12.0	$\frac{N}{100}$	$\frac{1}{1000000000000}$
	pH 13.0	$\frac{N}{10}$	$\frac{1}{10000000000000}$
	pH 14.0	$\frac{N}{1}$	$\frac{1}{100000000000000}$

Indicators.—An indicator is a substance which in the presence of certain materials in solution assumes a distinct color varying with the amount of acid or alkali present; or which changes sharply from one color to another as the reaction of the solution which contains it is changed.

[1] These mixtures retain their values for three or four weeks. For their proper use, see Newer Indicators.

[2] pH (potential hydrogen) as used by Sörensen represents the logarithm (to the base 10) of the reciprocal of the hydrogen-ion concentration. The mathematical equation is usually written, $pH = \log 10\frac{1}{H}$.

[3] Jour. Bacteriology, 1922, 7, 589. [4] A. L. Wachter: Personal Communication.

[5] The pH values given as the points of color change for the indicators recorded are, of course, only approximations. In the chart, as a whole, absolute accuracy has been sacrificed for simplicity of presentation.

[6] Ch is the symbol used for "concentration of hydrogen."

[7] At boiling temperature. At room temperature, "the first color change in clear solutions is at pH 8.0" (Clark and Lubs).

The chief indicators used in bacteriological work are litmus, phenolphthalein, the newer indicators such as brom thymol blue, brom cresol purple, phenol red, etc. (see p. 106); the anilin dyes and acid dyes as used by Andrade and Endo respectively (p. 110); and insoluble carbonates (p. 110).

Uses of Indicators.—Indicators are used in three ways:

1. To determine reaction of a culture medium in order to adjust it during the process of preparation.

2. To determine the end reactions of culture media either after sterilization and before being planted with bacteria, or after bacterial growth, for example in toxin production.

3. To be incorporated in culture media to indicate changing reactions due to growing bacteria.

1. **Determination and Adjustment of Reaction.**—In the determination of the true reaction and careful adjustment of culture media lies much of the success of media-making. It is necessary here to take into account the fact that media which are adjusted to a definite reaction before sterilization are found frequently to have changed somewhat during this process of heating. This is true particularly of those media made with fresh meat as a basis (Anthony and Ekroth.[1]) The effect of the heat applied in sterilization, especially that of the autoclave, tends to render them still more acid, due to the hydrolysis occurring in some of the ingredients. After a little experience one can learn to make allowance for this change in reaction, and, by the correct overadjustment of the reaction with sodium hydroxide[2] during the preparation of a medium, obtain the final reaction desired after sterilization. In accurate work, the final reaction should be determined on a sample of the finished product. The range in which most pathogenic bacteria (with several notable exceptions[3]) grow best, is between pH 7 and pH 8.

Litmus.—Litmus was the first indicator used in bacteriological work and has given valuable service because its change of color (from red in the presence of acid to blue in the presence of alkali) is at about pH 6.8, very near that of true neutrality or pH 7 (p. 107). However litmus is not accurate in H-ion concentration measurements, partly because it is not a definite compound and partly because azolitmin, its chief component, even though chemically pure, varies in its color reactions according to the material present in the solution being tested.

It is used chiefly as *litmus paper*, red and blue. By dipping these strips of paper in the medium to be tested we can determine roughly the reaction and adjust it either to neutral or slightly alkaline to litmus by adding a 4 per cent. solution of sodium hydroxide and retesting with the litmus paper. The red strip should turn slightly blue or the blue strip just a tinge less blue. *Litmus solution* (p. 110) may be used by adding 0.5 c.c. of a 5 per cent. solution to 5 c.c. of medium in a test-tube. Proceed as in Titrimetric Method given under Phenolphthalein (see below).

Phenolphthalein.—The color change of phenolphthalein, delicate pink to red, occurs at about pH 8.2 to pH 10. As this range is consid-

[1] Jour. Bacteriol., 1916, **1**, 209.

[2] When sodium carbonate is used for adjustment, the medium tends to become more alkaline when heated.

[3] B. tuberculosis, B. mallei, B. pertussis, pH5.

erably on the alkaline side of true neutrality, this indicator is colorless in acid solutions. Its use involves the operation called *titration*.

The materials necessary are a burette held in a clamp on a ring-stand, casserole and stirring rod, normal and twentieth normal sodium hydroxide and *phenolphthalein solution* (5 grams of the commercial salt to a liter of 50 per cent. alcohol.

Two methods may be used: (*a*) Room temperature (standard method) and (*b*) boiling temperature. The former should be used when the medium to be tested has been heated previously to the boiling-point during its preparation (p. 98, under Method II); with this method the reaction of a medium is set at a temperature more nearly approximating that at which it will be used, 37° C. of the incubator.

It is convenient, however, to use method (*b*) (boiling temperature) when titrating meat juice which has not been heated above 50° C. (p. 98, under Method I) for dissolving the added peptone and salt. Here the boiling temperature method approximates roughly the future conditions when the medium is boiled and sterilized. It has several serious objections, especially when it is misapplied: (1) The indicator phenolphthalein is less sensitive at the boiling-point and therefore less accurate. When a finished medium, prepared from fresh meat according to Method II (p. 98) and adjusted wrongly by the boiling temperature method, is tested by the room temperature method it will be found anywhere from 0.4 to 0.8 more alkaline than indicated by the boiling method. (2) The oft-quoted reason of ridding the mixture in the casserole of carbon dioxide is fallacious. If the sample is taken from a kettleful of medium boiling on the stove it contains little or no CO_2. The use of freshly boiled and cooled distilled water for the dilution (see below) eliminates still further the possible presence of CO_2.

Technic of Room Temperature Method.—Put 45 c.c. distilled water in a casserole. Add 1 c.c. phenolphthalein solution and 5 c.c. of the medium to be tested. (When titrating agar, warm the distilled water to 35° or 40° C., not above.) If no pink color is present the medium is acid to phenolphthalein. Read the burette, which contains twentieth normal sodium hydroxide (N/20 NaOH) and record the figures. While the mixture in the casserole is being stirred, run in a little of the solution from the burette very slowly until a faint pink[1] tinge is observed. This color should not disappear on stirring.

Read the burette again and record figures. Determine amount of N/20 NaOH withdrawn from the burette. On this is based the calculation for the adjustment of the reaction of the medium.

Calculation.—Five c.c. of medium required 2.4 c.c. of N/20 NaOH, therefore 100 c.c. (twenty times as much) would require 2.4 c.c. of N/1 NaOH (twenty times as strong); in other words, the medium is 2.4 per cent. acid to phenolphthalein or +2.4 if expressed according to *Fuller's method* or *scale*. Assuming that we desire a reaction of +1 per cent., we must then add 2.4 − 1 c.c. or 1.4 c.c. of N/1 NaOH to every 100 c.c. of medium or 14 c.c. to a liter.

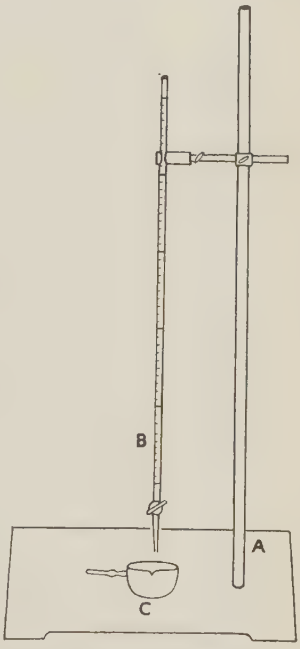


FIG. 26.—*A*, ring stand; *B*, burette; *C*, casserole.

[1] The pink may be described as a combination of 25 per cent. red and 75 per cent. white as shown by the disks of the standard color top.

If, on the other hand, the mixture in the casserole should show a pink color when the phenolphthalein is first added the medium is alkaline to this indicator. To determine the degree of alkalinity by the titrimetric method twentieth normal hydrochloric acid (N/20 HCl) is used in a burette and the same procedure followed as given above.

In the calculation if we use 0.5 c.c. of N/20 HCl then the medium is 0.5 (−0.5 per cent.) alkaline to phenolphthalein (*Fuller's scale*) and will require 0.5 c.c. of N/1 HCl for every 100 c.c. of medium or 5 c.c. for each liter. But if we wish the reaction to be + 1 or 1 per cent. acid, we must add 5 c.c. + 10 c.c. or 15 c.c. of N/1 HCl to each liter.

However, the addition of hydrochloric acid to a medium at any time is not at all to be recommended, as it may affect the growth of sensitive organisms. As a matter of fact, few media are naturally alkaline to phenolphthalein before adjustment. Such a condition occurs usually when an incorrect amount of alkali has been added in the attempt to reduce the natural acidity. The reduction of the natural acidity of a medium is the wisest procedure to follow, and it has been found far better to discard a medium which is too alkaline rather than to attempt to correct the error which has been made.

The reaction of a medium should be determined and adjusted at either *room temperature* or not above one approximating that at which the medium is to be used (37° C. of the incubator). In using phenolphthalein for titrating meat juice (p. 98 under Method I) which has not been heated above 50° C. for dissolving the added peptone and salt, the sample in the casserole is boiled for one minute as a *rough test* of the future conditions when the whole kettleful of medium is boiled. This is the only correct use of the *boiling method of titration.* It has several serious objections, especially when it is misapplied (p. 105).

Errors of the Titrimetric Method.—As stated above, the "acidity" of the medium is based on the amount of N/20 NaOH withdrawn from the burette. In adjusting the reaction of a medium to "neutral to phenolphthalein" (about pH 8, the point of first color change of this indicator) this procedure is fairly accurate, for in this case the whole amount of calculated N/1 NaOH is added. If, however, we attempt to use the Fuller method or scale and adjust, for example to + 1 or "1 per cent. acid to phenolphthalein" by adding only a part of the calculated amount of N/1 NaOH, we fall into error. To be sure, we have lessened the acidity by adding a certain amount of N/1 NaOH; but, because of the presence of an unknown amount of buffer substances (which combine with a part of the alkali added), we do not know how much acid has been neutralized nor the actual acidity of the medium when we have finished, unless we apply the colorimetric or electrometric method at this point.

In other words, the titrimetric method takes into account the total or titrable acidity of a solution and gives no data as to the actual acidity.

Newer Indicators[1] (for use in the colorimetric method).—For bacteriological work the most useful of these are:

		pH range.
Brom cresol purple[2]	yellow-purple	5.2–6.8
Brom thymol blue[2]	yellow-blue	6.0–7.6
Phenol red[2]	yellow-red.	6.8–8.4

The ranges of most of these indicators overlap one another (see Chart p. 107). In making up the solutions[3] of these indicators use 0.04 per cent. in 95 per

[1] A full list covering the whole range of pH 1 to pH 10 will be found in the article by Clark and Lubs.

[2] In ordering these indicators use the chemical names as follows:
Di bromo ortho cresol sulfon phthalein (brom cresol purple).
Di bromo thymol sulfon phthalein (brom thymol blue).
Phenol sulphon phthalein (phenol red).

[3] Report of Committee on Chart, etc., Jour. Bacteriol., 1918, **3**, 2.

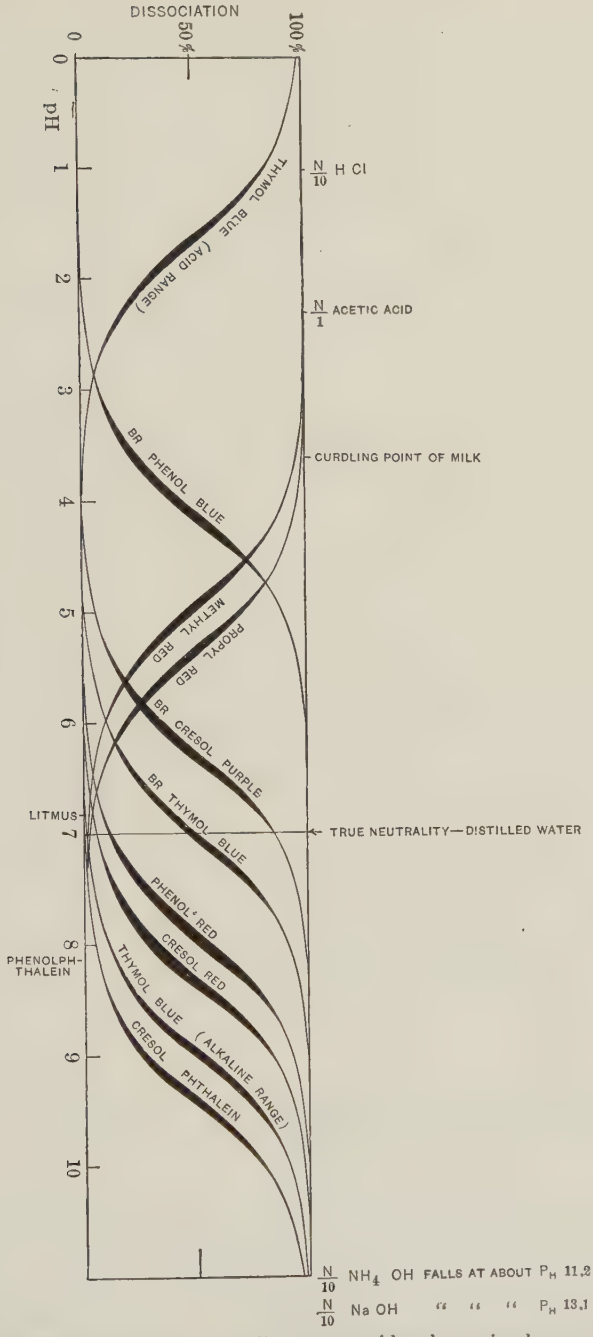


FIG. 27.—Dissociation curves of indicators considered as simple monobasic acids, showing percentage color change with pH. Shaded portions of curves indicate the useful ranges. (Clark and Lub's chart modified.)

cent. alcohol of the brom cresol purple and brom thymol blue; of the phenol red, use 0.02 per cent.

The materials needed are test-tubes of clear glass and uniform diameter (6 by $\frac{3}{4}$ inches is a convenient size), freshly distilled water (ammonia-free,) normal and twentieth normal sodium hydroxide solutions,[1] burette, comparator block (p. 108) and tubes of the standard solutions of known hydrogen-ion concentration, each tube containing a suitable indicator in a definite amount, for example, 0.25 c.c.

Procedure of Determining Actual Reaction by Colorimetric Method.—Put 2 c.c. of the medium to be tested and 8 c.c. of distilled water in each of two test-tubes. (If agar is being tested, warm the water to 35° to 40° C., not above). To one tube add 0.25 c.c. (or same amount as in the standard tubes) of an indicator whose range covers the probable H-ion concentration (actual acidity) of the medium, for example pH 6. Compare this tube with the standard tube marked pH 6. The comparison should be made in the comparator block (p. 109) in which the color of the medium in the tube without indicator is superimposed on the standard tube. If the tube of medium plus indicator matches the standard tube pH 6, the reaction of the medium is pH 6; if the shade of color falls midway between the standard tubes pH 6 and pH 6.2, the reaction is read as pH 6.1. If the color matches neither *exactly*, read to the nearest.

If, however, the wrong indicator has been chosen, the color will be either too intense a purple, blue or red (depending on the indicator chosen) or an intense yellow, showing that the limits of the range have been exceeded. In either case prepare a new tube of medium diluted as before and try another indicator.[2]

Procedure of Adjustment of Reaction Colorimetric Method.—Prepare two tubes of medium diluted as directed under Determining Actual Reaction. To one tube add 0.25 c.c. (or same amount as in the standard tube) of an indicator whose range covers the desired H-ion concentration, for example, pH 7.4. Place the tubes in the comparator block and compare with the standard tube pH 7.4 containing the same indicator in the same amount. If the shades of color are not the same and the media tube is found to have a pH value less than 7.4, add to it N/20 NaOH, drop by drop, from a burette, mixing the contents of the tube after each addition of alkali until the shade of color matches that of the chosen standard tube (pH 7.4).

Calculation.—25 × the amount of N/20 NaOH withdrawn from the burette gives the amount of N/1 NaOH to be added to 1 liter of the medium when 2 c.c. are tested.

Example: If 2 c.c. of medium required 0.3 c.c. of N/20 NaOH, 5 c.c. of medium (two and a half times as much) would require $2.5 \times 0.3 = 0.75$ c.c.; therefore, 100 c.c. (twenty times as much) would require 0.75 c.c. of N/1 NaOH (twenty times as strong) and 1000 c.c. would require 7.5 c.c. or $0.3 \times 25 = 7.5$ c.c.

Comparator Block.—The use of a comparator block is very helpful, especially when the material to be tested is either colored or turbid. The block can be easily constructed by boring holes, as in diagram, for the test-tubes in a piece 2 x 4 inches. Slits or other holes are then cut

[1] N/1 NaOH and N/20 are not essential for this test. A 4 per cent. solution of NaOH (approximately normal) may be used. For the burette, dilute the 4 per cent. NaOH accurately to make a twentieth dilution. (Put 5 c.c. of 4 per cent NaOH in a 100 c.c. volumetric flask and fill up to 100 c.c. with distilled water.)

[2] To save time, preliminary tests may be made: Place several large, well separated drops of the diluted medium on a porcelain plate. To each drop, add one drop of a different indicator. The resulting colors will serve as guides in the choice of the correct indicator to use for the test. This procedure is called "spotting."

in the opposite sides so that the tubes can be viewed in pairs from the side, and are arranged as follows:

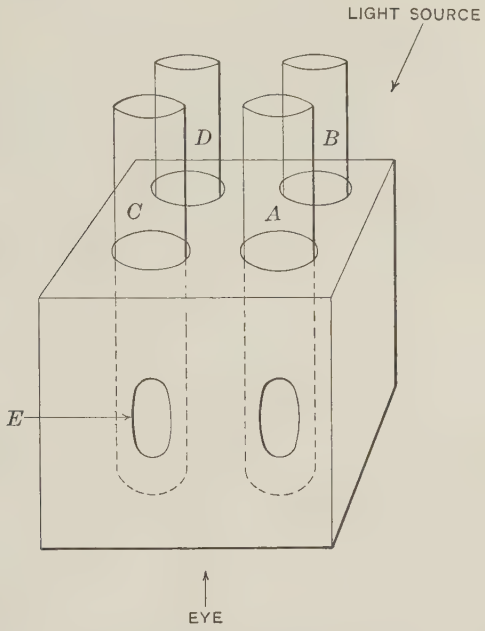


FIG. 28.—Comparator Block: *A*, medium + indicator; *B*, water; *C*, medium + no indicator; *D*, standard solution + indicator; *E*, slit for observation.

A comparator block for the Medalia "color standards" set requires three sets of three holes each in order to view the tubes in threes.

2. **Determination of End Reaction of Culture Media after Bacterial Growth.**—The degree of alkalinity or acidity produced in culture media after bacterial growth is most accurately determined by the electrometric or colorimetric methods. The fallacies of the titrimetric method have been pointed out on p. 106. See also actual acidity and total acidity (p. 101).

Either the whole culture or a portion of it may be subjected to the tests as given above.

METHYL RED is used to differentiate between the colon and aërogenes cloacæ groups. The former group gives a distinct red color and the latter a distinct yellow color with this indicator. Prepare the methyl red solution by dissolving 0.1 gram in 300 c.c. alcohol and dilute to 500 c.c. with distilled water. Add 5 drops of this solution to each 5 c.c. of culture.

The culture medium, dextrose phosphate broth, for this test consists of 800 c.c. distilled water, 5 grams of Witte peptone (no other), 5 grams c.p. dextrose and 5 grams K_2HPO_4. Heat over steam with occasional stirring for twenty minutes. Filter through paper, cool to 20° C. and dilute to 1000 c.c. with distilled water. Put 10 c.c. in each test-tube. Sterilize in an Arnold sterilizer for twenty minutes on three successive days.

3. **Indicators Incorporated in Finished Media to Indicate Changing in Reactions Due to Growing Bacteria.**—LITMUS (see also p. 104).—Litmus for this purpose

is in the form of a solution (see below). The reaction of the medium to which it is added must be neutral (or very slightly alkaline) to this indicator. The disadvantage of litmus is that it may be decolorized by the growth of bacteria which rob it of its oxygen and reduce it to the colorless leucobase. The color will return on exposure to the air. When stab cultures are used it may be necessary to melt the medium and pour it into Petri dishes to get sufficient air to cause the color to return. If the color change of individual colonies is to be observed the diffusion of any acid produced may be prevented by using 3 per cent. agar instead of 1.5 per cent.

Litmus Solution.—A purified litmus (Merck or Kahlbaum) which comes in dry form is pulverized and added to distilled water in a 5 per cent. amount. Steam in an Arnold sterilizer for two hours, shaking the mixture every twenty minutes. The solution is then filtered and sterilized in the Arnold preferably in small amounts for convenience as it must be kept sterile. Usually 5 per cent. of the solution is added to a medium, but this may be varied to suit individual preferences.

Phenolphthalein.—The medium should be faintly alkaline to this indicator and sufficient 1 per cent. alcoholic solution added to give a faint distinct pink color. The color is discharged when acid is produced.

Newer Indicators (p. 106).—For more definite determination of the changes in reaction one or more of the newer indicators should be used. Brom cresol purple, phenol red and cresol red are perhaps the most useful. (It is to be noted that brom thymol blue gives unsatisfactory results in milk.) The choice depends chiefly on the initial reaction of the finished medium. That indicator should be selected whose range either covers the initial reaction (for example, pH 7) or whose range is slightly to the acid side of the initial reaction: for example, brom cresol purple (pH 5.2 to 6.8). As the bacterial growth proceeds, changes in the pH value of the medium may be watched and recorded from day to day.

The above indicators are not reduced by bacterial growth (as are litmus and methyl red) nor do they themselves, in the minute quantities necessary, affect bacterial growth. The amount of indicator used in the medium is not important so long as the color obtained is distinct. A convenient strength has been found to be 1.6 per cent. alcoholic solution. Add 1 c.c. of this to 1 liter of medium. It must be recalled, however, that in making comparisons with the standard tubes (p. 102 and 107), proportionate amounts of the indicator should be present in the standards. These indicators are not affected by sterilization in the autoclave.

Various anilin dyes which are reduced to their colorless leukobase by the action of sodium sulphite (Endo) or acid dyes decolorized by sodium hydroxide (Andrade[1]) may be used as indicators. When the sugar is split by the bacteria, aldehydes and organic acids are produced, which cause the color to return. The former act most vigorously on the Endo indicator. (See Typhoid Media.) Media to be used with the Endo indicator should be about pH 8.6 or 8.8, alkaline to phenolphthalein. The Andrade indicator consists of 100 c.c. of a 0.5 per cent. water solution of acid fuchsin decolorized by the addition of 16 c.c. N/1 NaOH solution. One per cent. is added to the media. The reaction of the medium is adjusted to the indicator so that it is distinctly pink when hot but colorless when cold. That is, make the basic medium about pH 6.9.

Powdered Insoluble Carbonates may be used in solid plating media. If the powder is evenly distributed throughout the media, acid-producing colonies will be surrounded by a clear area.

In general media containing indicators are sterilized preferably in an Arnold sterilizer. The autoclave may be used in emergencies, but the indicator is somewhat injured.

Clearing of Media.—This is done by the coagulation of an albumin by heat (autoclave, Arnold or by boiling). As the albumin coagulates

[1] Jour. Infect. Dis., 1914, **15**, 227.

it enmeshes the fine particles floating in the medium and the whole mass is later removed by filtration. The albumin may be either that present naturally, as in meat infusion (soluble albumin, p. 95), or added materials as eggs, either fresh or in the form of dried egg albumen. The hot medium must be cooled to about 50° C. before the egg is added.

If fresh eggs are used one is allowed to each liter. After breaking the egg in a small pan it is mixed with an equal amount of water by means of an egg beater. The mixture is then added to the cooled medium and the whole stirred thoroughly. When dried egg albumen is used 10 grams are added to 20 c.c. of water and allowed to stand overnight to dissolve thoroughly. Add the 20 c.c. to 1 liter of medium as above.

The medium is then heated usually in an autoclave at 10 to 15 pounds' pressure for thirty minutes or in an Arnold sterilizer for forty-five to sixty minutes. Boiling over the free flame is not so satisfactory.

Filtration of Media. — For *fluids* use filter paper reinforced with a piece of cotton at the neck of a glass funnel. The paper used should be moistened with cold water to prevent the passage of any fats present in the medium.

Media which solidify on cooling (agar and gelatin) are usually filtered satisfactorily through absorbent cotton in a glass funnel kept warm by a hot-water jacket (Fig. 29). However, the use of paper-pulp in a Buchner funnel (Fig. 30) or of a Sharples centrifuge is desirable at times to produce a very clear medium. On the other hand mere sedimentation may be all that is necessary or advisable. (See Vitamin Agar.)

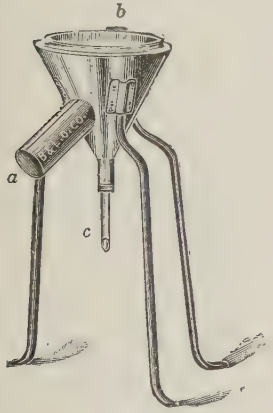


FIG. 29.—Hot-water funnel or jacket: *a*, point of heat; *b*, inlet for water; *c*, glass funnel. The water is kept hot by placing flame of Bunsen burner under *a*.

FIG. 30.—Buchner funnel.

Preparation and Use of Funnels.—A good grade of absorbent cotton with long fibers is essential so that the usual layer of cotton may be split horizontally. Lay the split portions crosswise in a glass funnel in which has been placed a small square of coarse wire netting, about one-quarter inch mesh, such as wire baskets are made of. This prevents the cotton from jamming in the neck of the funnel and also gives a broad dripping surface. The glass funnel should be placed in a *hot-filtration funnel* (Fig. 29) or kept warm in an Arnold sterilizer.

Moisten the ends of the cotton with some of the medium so they will adhere to the glass, then carefully moisten the rest of the cotton with a dipperful of medium. Add small portions of the medium slowly at first. The coagulated egg and albumins which settle on the cotton act as a part of the filter. Pour back the filtrate several times until it comes through clear. The flasks in which it is received should be warmed by rinsing with hot water. The rest of the medium should be kept hot in the Arnold sterilizer until it is put in the filter.

Paper-pulp in Buchner Funnel.—The nine-inch size Buchner funnel is suitable for media in 5- to 10-liter lots. It is used with a suction pump. The ordinary filtration pump (p. 118) which gives about eleven inches' vacuum is sufficient. *Paper Pulp.*—Prepare paper-pulp by soaking scraps of ordinary filter paper for thirty-six to forty-eight hours in hot water in a large wide-mouthed bottle. The paper and water should be in the proportion of 6 sheets of filter paper (20 x 20 inches) to 2½ liters of water. Shake the mixture vigorously at intervals to make the suspension fine and uniform. When ready to prepare the 9-inch funnel, dilute 400 to 500 c.c. of the pulp with about 3 liters of hot water. Cut a piece of surgeons' lint (or cotton flannel) to fit the bottom of the funnel exactly. Rinse the funnel with hot water. Place in it the lint, fleecy side uppermost. Pour in the hot paper-pulp suspension carefully so as to cover the lint with an even layer about one-eighth to one-fourth inch thick. Over this lay a disk of filter paper. Place a 4-liter suction flask under the funnel and apply the suction pump to draw the water into the filtration flask until the pulp is firm, yet somewhat moist. The agar will not go through too dry a filter.

The funnel and paper-pulp must be hot when the agar is poured in carefully and slowly, striking the disk of filter paper which prevents the breaking of the surface of the pulp. Discard the first 100 c.c. of agar which come through for it contains some of the water from the pulp.

Media filtered by this method must *not* have egg added for clearing. It clogs the filter.

The **Sharples Super-centrifuge** works on the principle of the milk separator. The machinery is very complicated, but it gives excellent results.

Sedimentation.—With this method the agar medium is allowed to cool slowly, so that the larger particles may settle at the bottom of a straight-sided container. After it has stood overnight the hardened mass is turned out as from a jelly mould and the sediment trimmed away from the clearer portion. The fine particles remain in the medium. By sedimentation, or by use of a Sharples centrifuge, all contact of the medium with paper or cotton may be avoided and the "vitamins" (p. 128) retained.

Preparation of Media for Use.—After filtration the medium may be sterilized (p. 113) either in flasks for storage or in various containers suitable for immediate use—such as test tubes and bottles. In filling any container avoid wetting the neck of it or the cotton plug will stick. Use a glass funnel with a stop-cock; or fit rubber tubing, with a piece of glass in one end, on an ordinary funnel and control the outflow with a pinch-cock (Fig. 43).

The amount of medium in a container depends on the use to which it will be put. For the ordinary 15 cm. (six-inch) test-tube a depth of 3 to 5 cm. is sufficient. If plates are to be poured at least 10 c.c. are necessary, but if the medium is to be slanted, 6 to 7 c.c. suffice. The slants may be made either immediately after sterilization or the medium may be remelted and slanted as required.

It is convenient to remelt sterilized media by immersing the tubes, held in a wire basket, in a small kettle of water which should rise above the level of the medium. The water is then brought to the boiling-point and kept there until the medium is melted. The flame should then be lowered and the medium removed as promptly as possible from the influence of the high temperature.

Prolonged or repeated heating of a medium tends to change the reaction and also reduces the solidifying qualities of agar as well as of gelatin. For bottles, flasks or large numbers of tubes the Arnold sterilizer may be used for remelting.

To make the slants, place the tubes after sterilization in a row on a table with a glass rod or strip of wood to raise the upper ends. Care should be taken that no slant touches the cotton and that sufficient medium be present in the bottom of the tube ("butt") to prevent the slant from collapsing when the tube is raised. The medium should be well set before the tubes are moved.

Fermentation Tubes (Fig. 21).—Fermentation tubes are used for liquid media only. In filling types "a" and "b" the tube must be tilted until no air-bubble remains in the closed arm. With type "c" the small inner tube, which is separate, is filled first, then a small amount of medium is run in the large tube. Slip the small tube, with as little inversion as possible, mouth down into the large tube. There should be no air-bubble in the smaller tube. Practice is needed for this operation. Add more medium if necessary to the larger tube and replace plug.

All fermentation tubes must be sterilized in the Arnold sterilizer (p. 115).

Sterilization of Media. — Sterilization may be accomplished by *heat, filtration* or by the use of *chemicals*.

Heat.—The heat may be applied in one of several ways: (1) Steam under pressure (autoclave); (2) streaming steam (Arnold); (3) water-

Fig. 31.—Upright type.

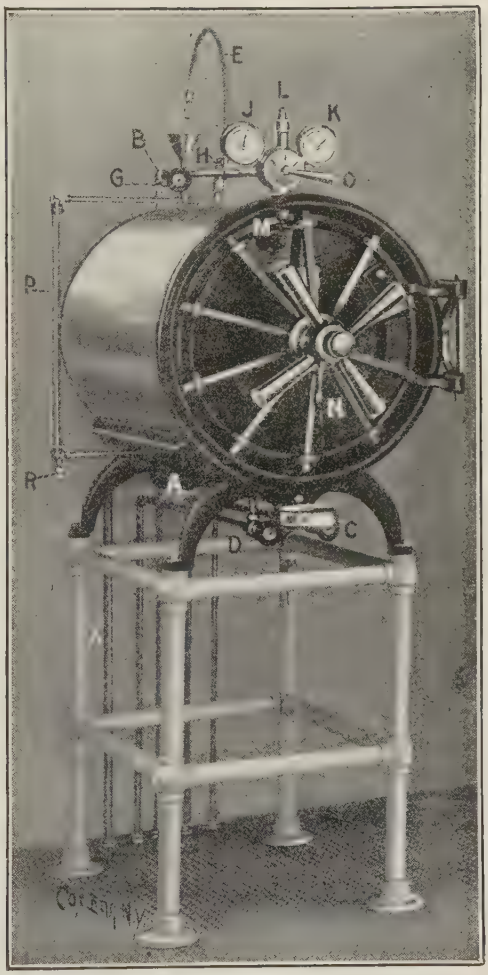


Fig. 32.—Horizontal type.

Figs. 31 and 32.—Autoclaves may be heated by direct application of heat, or when available, by steam under pressure.

bath. Methods (2) and (3) are usually known as intermittent ("fractional") sterilization.

1. **Steam under Pressure.**—The sterilization is accomplished by means of an autoclave, various forms of which are available (Figs. 31 and 32). They may be heated either by gas or steam from the boiler-room. All air in the chamber should be displaced by the steam before closing the pet-cock or vent in order to avoid air-pockets which interfere with thorough sterilization. In the simplest

form (Figs. 31 and 33) the water in the bottom is brought to the boiling-point after the lid is clamped down. The displacement of any air in the chamber is accomplished by allowing the vent to remain open until steam has issued for a minute or two. The steam is then brought up to the desired pressure, as shown by the pressure-gauge, by continued heating. A safety valve set to the required pressure releases a portion of the steam when that pressure is reached. In the more complex type (Figs. 32 and 34) the water in the surrounding jacket is heated and part of it converted into steam until the pressure in the jacket exceeds by several pounds the pressure desired in the chamber. By a system of valves the steam from the jacket is then allowed to fill the

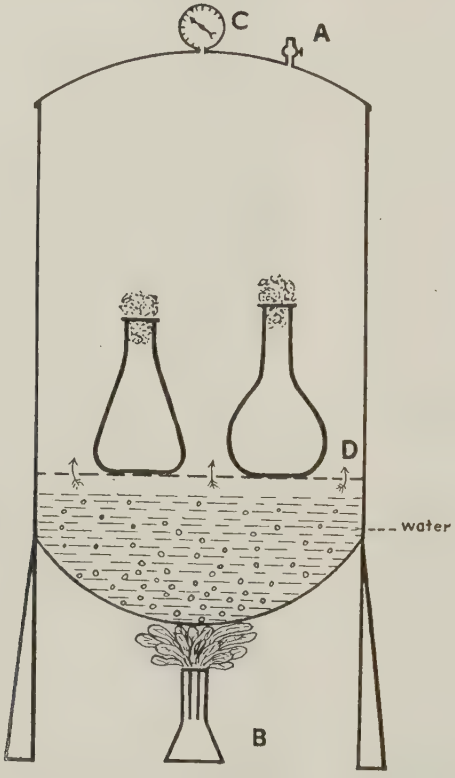


FIG. 33.—Cross-section of simple upright autoclave. *A*, pet-cock or vent; *B*, source of heat; *C*, dial; *D*, steam.

chamber and, after displacement of the air as above, the vent is closed and the pressure in the chamber reaches the required point. This pressure is controlled by a safety valve (*L*, Fig. 32).

At the end of the "run" the steam supply is shut off and the pressure allowed to decrease gradually by opening the vent a very little. Too rapid a reduction of the pressure will cause the media to boil up and wet or even blow out the plugs.

The pressure and time employed depend on conditions. As a rule, 15 pounds' pressure is employed and twenty minutes is sufficient for media in test-tubes. Flasks should be heated one-half to one hour, depending on their size. Lower pressures are used at times when overheating is undesirable, and the heating repeated on two successive days. The heating should be calculated from the

time the desired pressure is reached. The temperature will vary with the pressure thus:

5	pounds'	pressure	= temperature	108.8° C.
10	"	"	= "	115.6° C.
15	"	"	= "	121.3° C.
17	"	"	= "	123.3° C.
20	"	"	= "	126.2° C.
24	"	"	= "	129.3° C.
30	"	"	= "	134.6° C.

Since the pressure in the autoclave may fluctuate it is desirable to use a recording pressure-gauge which shows graphically on a chart the period of the run and the pressure maintained (Fig. 35). These charts may be dated and filed for reference.

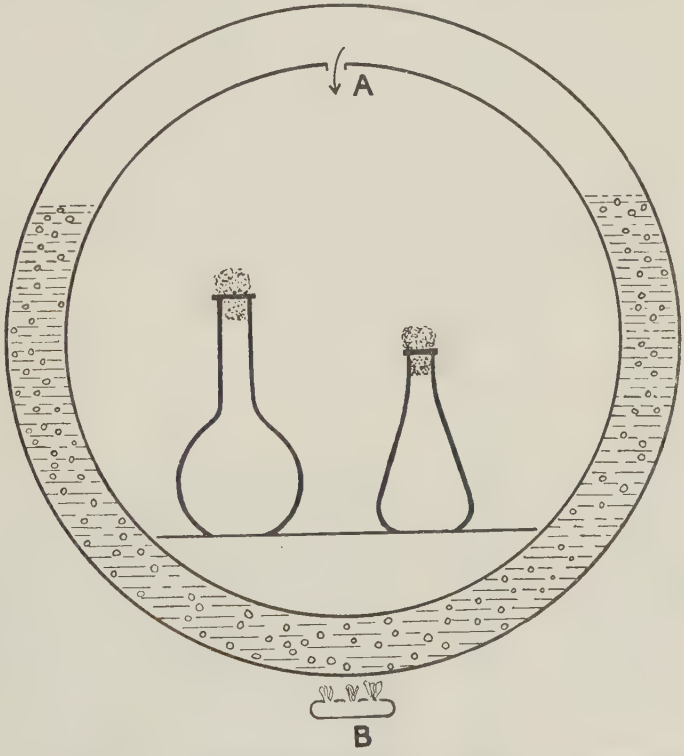


FIG. 34.—Diagram of inner construction of horizontal autoclave. *A*, passage for steam from jacket to chamber; *B*, source of heat if gas is used.

Agar media must be in the liquid state when put in the autoclave for sterilization or time for melting must be allowed before calculating the time of sterilization.

2. **Streaming Steam.**—Streaming steam is applied by means of an Arnold sterilizer (Figs. 36 and 37). By this means a temperature of 100° C, or close to it is applied to media containing special sugars, etc. This heating must be repeated on three or more successive days, the daily period depending on the size of the container; test-tubes of media require twenty minutes, whereas 1- to 2-liter flasks require forty-five to sixty minutes to allow the heat to penetrate. With agar media the period is timed from the minute the agar is wholly melted.

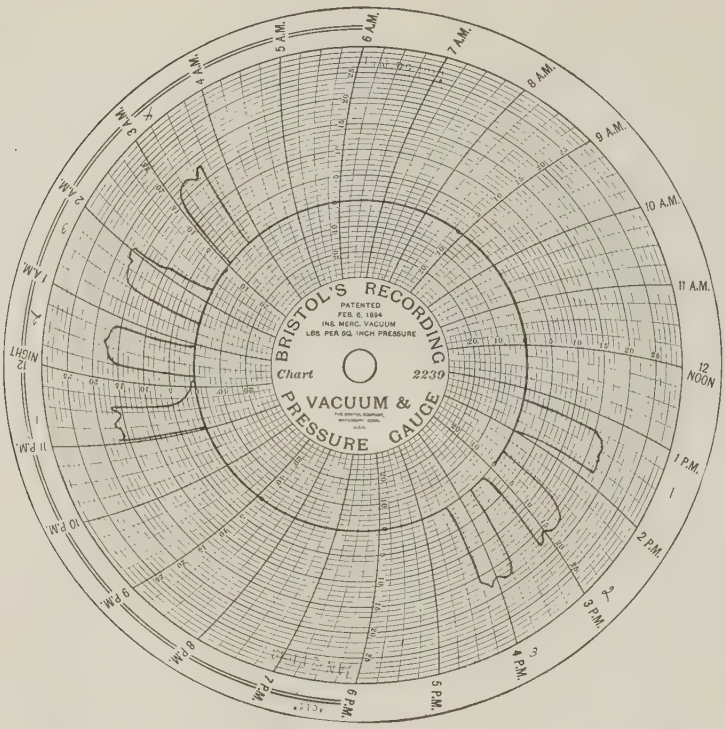


FIG. 35.—Recording chart of autoclave.

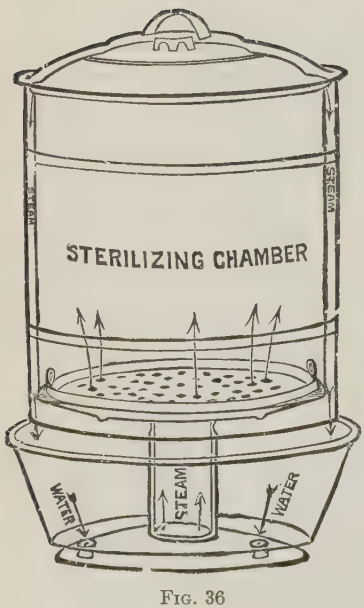


FIG. 36

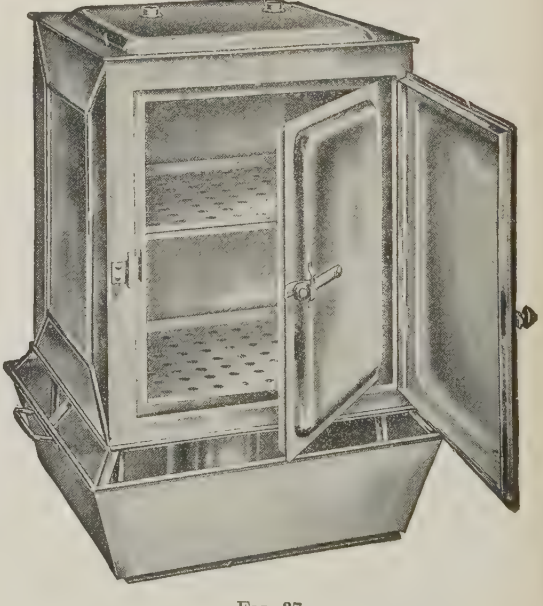


FIG. 37

FIGS. 36 and 37.—Arnold steam sterilizer. Two types.

3. **Water-bath.**—In this method temperatures of 60° to 80° C. are applied on successive days to such substances as blood serum, transudates from body cavities (*e. g.*, ascitic fluid) etc.

As stated above, methods (2) and (3) are known as intermittent (or "fractional") sterilization.

The principle underlying *fractional* or *intermittent* sterilization is as follows: All bacteria when free from spores are killed by exposure for one or two hours to only 60° to 80° C. heat. If the material is left overnight at about 22° C., spores, if present, will develop into bacteria and be destroyed by the second heating. Some of the bacteria may produce spores before the second heating or some of the spores may be slow in developing and escape. For this reason a third heating, or with the low temperatures, five or six heatings on successive days may be necessary.

When method (3) is applied to albuminous material before coagulation, such as sera, tissue extracts, etc., the temperature selected should be below the coagulation-point of the material, usually about 60° C. All material to be treated in this manner should be as free from contamination as possible. The method has its best application to originally sterile material which, because of necessary manipulation, may have become slightly contaminated. The heating may be done in a water-bath or in a water-jacket oven.

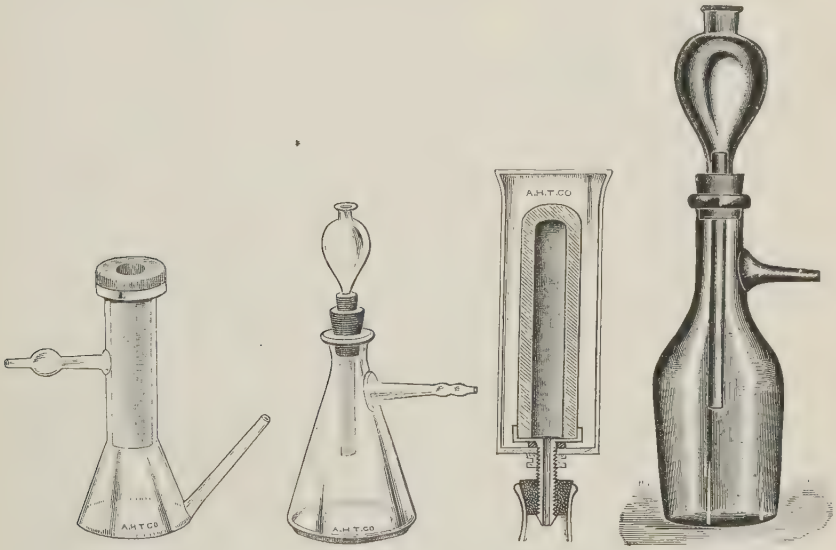


Fig. 38 Fig. 39 Fig. 40 Fig. 41

Figs. 38, 39, 40 and 41.—Types of filters.

Filtration.—This method is resorted to when the application of heat will injure the solution as in the case of some sugars, or when the necessary amount of heat would cause coagulation, as in the case of tissue extracts or serum. Various types of filters made of unglazed porcelain or compressed diatomaceous earth are used. Types of these filters are the Berkefeld, Pasteur, Chamberland, Mandler and Doulton filters. A new form of filter is offered by Haen which is in the form of a membrane. The bacteria are held back because of the fineness of the pores. Various grades of fineness are procurable in some brands, and on this depends the rapidity of filtration. The filter candle and all attachments with which the fluid will come in contact after passage through the candle must be sterile. The glassware can be sterilized in the hot-air oven and the filter candle and rubber connections can be sterilized by boiling for one hour or

by steam under pressure. (See Autoclave.) After use the filter candle should be freed of all soluble material, especially coagulated matter, by running through it an abundance of clear water. If used for infective matter, it can then be sterilized by boiling. In any case the surface should be lightly scrubbed with a fine brush after use.

A new filter should be cleansed before use by filtering clear water. It should then be placed in cold water and boiled thoroughly.

After continued use the candles gradually become clogged. They can be renewed to some degree by careful heating to glowing in an oven. This is apt to produce fissures, hence the filter should be retested before use.

In any case the permeability of the filter should be tested before use by filtering a broth culture of some small microörganism, such as Bacillus prodigiosus.

Before filtration the fluid to be filtered should be rendered as clear as possible by passing it through paper or if necessary through paper-pulp.

Coarse filtration is accomplished by using paper-pulp or sand. The paper-pulp is prepared by soaking torn-up filter paper in water and then placing a layer on the perforated porcelain plate of a porcelain funnel, draining off the water and packing the layer tight. The depth of the layer will depend upon the fluid to be filtered, its density and the fineness of the particles to be removed. The funnel should be fastened in the neck of a filtering flask and suction applied to hasten the filtration. Similar results may be obtained by filtration through sand.

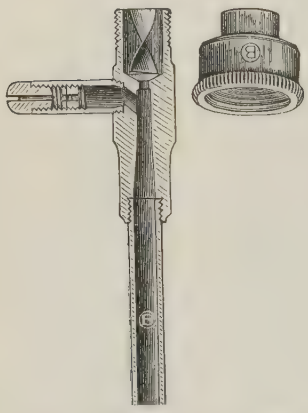


Fig. 42.—Filter pump for attachment to water faucet.

The filtration through a filter candle (the size selected will depend on the amount to be filtered) is accelerated by the use of suction or pressure. Suction may be secured by the use of a filter pump attached to the hydrant or other types of suction apparatus. Pressure may be obtained by air under pressure from an installed system or by a hand pump and cylinder to equalize the pressure. A manometer is employed to determine the pressure. The accompanying figures show the method of setting up the apparatus (Figs. 38 to 42).

Chemicals.—Chemicals such as chloroform are used occasionally, especially for the preservation of sera which may have suffered slight contamination.

Storage of Media.—After sterilization media may deteriorate for two reasons—contamination and drying. Contamination is due frequently to the penetration of the stopper by moulds. This may be prevented by capping the container with filter paper before sterilization. Rubber tissue tied over the neck of a flask, or rubber caps made for the purpose are also useful. These are applied after sterilization. A small pledget of cotton moistened with bichloride of mercury solution may be placed between the cap and the stopper to discourage the development of any moulds which may have adhered during the manipulation. The neck of the flask should be wiped free of the bichloride before pouring out the medium.

Drying may be avoided by capping as above or by sealing with paraffin or sealing wax. The plugs of tubes are dipped in hot paraffin. With flasks the plug is cut off, pushed in slightly and the melted paraffin poured on top. The paraffin should be nearly ready to set so that it will not run through the plug into the medium.

It is well to keep most media in a dry refrigerator at 40° F. to 45° F.

Formulæ and Uses of Culture Media.

Basic Liquid Media.—Meat infusion (nutrient) broth and its variant beef extract have been given on pp. 98 and 99.

Basic Solid Media.—Gelatin Media.—Meat infusion gelatin may be prepared in two ways: 1. To 1000 c.c. meat juice (p. 98 Method I) add gelatin, 100 grams (10 per cent.) together with peptone 10 grams, sodium chloride 0.5 gram. Follow the procedure of Method I. The coagulation of the albumins present in the meat juice clears the medium. Filter through cotton (p. 111), tube and sterilize[1] in the Arnold sterilizer twenty to thirty minutes on three successive days.

2. To 1000 c.c. *meat infusion* (p. 98, Method II) add peptone 10 grams (10 per cent.), sodium chloride 0.5 grams. Heat almost to the boiling point, add the gelatin, 100 grams, and dissolve with as little heating as possible. Set reaction (p. 99) neutral to litmus (or about pH 6.8 or pH 7.) Cool to 50° C. and clear with egg (p. 111) by heating for forty-five minutes in an Arnold sterilizer. Filter, tube and sterilize as above.

Beef Extract Gelatin.—Add 10 grams (10 per cent.) of gelatin to beef extract broth (p. 99). The gelatin dissolves readily at the boiling-point. Proceed as in meat infusion gelatin, second way.

Agar Media.—Meat Infusion Agar.—Any one of several methods may be used.

1. The simplest is to add the agar 1.5[2] per cent. to nutrient broth prepared by either Method I or Method II (p. 98). Dissolve the agar either by heating in the autoclave at 10 to 15 pounds for thirty minutes or by boiling over the free flame. In this case the water lost by evaporation should be made up by adding hot water. Set reaction (p. 99). Cool to 50° C. and add 1 egg for each liter (4 eggs for 5 liters). To clear (p. 111) heat in autoclave at 10 to 15 pounds for thirty minutes or Arnold sterilizer for one hour. Test the reaction and adjust if necessary. If more than 0.2 per cent. normal soda is required per liter, heat again for ten minutes to throw down any precipitate. Filter through cotton (p. 111). Fill tubes or flasks and sterilize at 15 pounds' pressure for thirty minutes.

2. The agar may be added to the meat juice (p. 98, Method I) after the peptone and sodium chloride have been dissolved. Proceed as above.

3. Add the agar to *meat infusion* (p. 98, Method II) together with the peptone and sodium chloride. Proceed as above under (1).

4. Double strength method: Make double strength meat juice (p. 98, Method I) or meat infusion (p. 98, Method II). Add double amounts of peptone and salt. Set the reaction. To an equal quantity of water add double the percentage of agar and dissolve it. Cool the agar below 50° C. and mix the two solutions. Test the reaction and adjust if necessary. Proceed as directed above under (2) or (3) depending upon the choice made as to meat juice or meat infusion.

Beef Extract Agar.—Beef extract agar may be made in several ways:

(1) Add 1.5 per cent. agar to 1000 c.c. of meat extract broth. Proceed as in meat infusion agar under method (1).

(2) Weigh out the ingredients as for beef extract broth and add 1.5 per cent. agar. Dissolve the whole by placing the mixture in autoclave, as above, or by boiling over the free flame. Proceed as in meat infusion agar under (1).

(3) Double Strength Method.—Make a double-strength beef extract broth. To an equal amount of water add double the percentage of agar. Dissolve the agar, cool to 50° C. and mix the two solutions. Proceed as in meat infusion agar under (1).

[1] Avoid overheating and additional sterilization as the medium will not set if heated too much.

[2] Two per cent. is better if other substances such as blood or serum are to be added after sterilization.

Semisolid Media.—Various types of semisolids are in use. The amount of agar will vary according to the use to which it is put. For slants, 0.75 per cent. to 1 per cent. of agar is necessary; for stab cultures, 0.5 per cent. or even less is sufficient. If the agar is to be diluted by the addition of serum or other enriching substance, this must be allowed for in preparing the medium. The following is an example (*North*): Meat infusion (1 pound of meat to 500 c.c. of water) is warmed and 20 grams of gelatin and 20 grams of peptone dissolved in it. In 500 c.c. of water dissolve 10 grams of agar and cool to below 50° C. Mix the two, adjust the reaction, and heat to coagulate the albumin and clear the medium. Readjust the reaction and reheat if necessary; filter.

A very satisfactory medium can be made by simply using 0.5 per cent. or less of agar instead of the usual 1.5 per cent. employed. This can be diluted by the addition of one-third of its bulk of an enrichment fluid. The finished medium should just "set" sufficiently for stab culture purposes. Nutrose (1 per cent.) may also be added.

Digestion Products in the Preparation of Media.—Martin's Peptone Solution.[1]—Five pigs' stomachs are cleaned, freed from fat and finely minced. (A number of stomachs should be used to equalize the peptone content; in this way an almost average composition in peptone is obtained.) Mixed stomachs (minced), 200 grams, hydrochloric acid (pure), 10 grams, water (at 50° C.), 1000 c.c. Keep at a temperature of 50° C. for twenty to twenty-four hours in a glass or porcelain vessel, *not* enamel. It is most important not to allow peptone to come in contact with any metal until it is neutralized. Heat to 80° C. to stop digestion. Pass through a layer of absorbent cotton. (At this point it can be stored without sterilization.) Heat filtrate to 70° C. and neutralize to litmus at this temperature. Sterilize in autoclave at 10 pounds' pressure for fifteen to twenty minutes and store away.

Martin's Peptone Broth.—To meat infusion add an equal amount of Martin's peptone which has been neutralized. The mixture is best made with both the peptone and broth at 70° C. Titrate (room temperature method) and set to desired reaction. Autoclave for fifteen minutes to clear. Titrate again and set reaction as before. Filter through paper and cotton and sterilize in autoclave at 15 pounds' pressure for one-half hour.

Digested Meat Media.—Meat, etc., digested by pancreatin, trypsin or other ferments.—Hottinger[2] makes the statement that the greater part of the nutritive elements of meat are lost in the usual process of making broth for laboratory use. To avoid this, a process of slow digestion is recommended, with the addition of pancreatin, and it is claimed that in this way a much more favorable medium can be obtained for the growth of bacteria, with so much peptone developed as a result of the meat digestion, that *no addition of commercial peptone is required.* Sodium chloride is also omitted.

Hottinger Broth (slightly modified).—Use 750 grams of meat to 1500 c.c. water. The meat, carefully freed from fascia, is cut in finger-thick pieces. Heat the water to boiling. Drop in the meat, piece by piece, stirring constantly. Boil up strongly and take from fire. Take out meat and put through chopping machine. Cool water to 37° C. and to it add sodium carbonate, 1.5 grams per liter. Put chopped meat in flasks (2-liter, Erlenmeyer) 550 grams per flask. Add the water (37° C.) to flasks, filling them up to narrow neck. To each flask add: pancreatin 3 grams, chloroform 10 c.c., toluol 10 c.c.

Cork tightly and shake well. Incubate at 37° C. overnight. Shake next day and add more pancreatin unless the fluid shows a yellow color and particles of meat look smaller, showing that digestion is taking place.

The process of digestion should continue for four or five days at room temperature or for two or three days in incubator; shake flasks each day. At the end of this time the meat has become a finely divided mass, giving off a very offensive odor. According to Hottinger the medium may be stored in the ice-box

[1] Ann. de l'Inst. Pasteur, 1898, **12**, 38.
[2] Centralbl. f. Bakteriol., 1912, **63**, 178.

at this point without heating again, after testing with litmus paper and acidifying with a slight amount of dilute HCl, if found to be alkaline. In our experience this plan has not worked well, and we have found it best to proceed at once as follows:

Decant liquid through cheese-cloth. Add an equal amount of water to the residue. Shake well. Allow meat to settle and again decant. Finally place meat on cheese-cloth and allow to drain. Boil the filtrate for a few minutes, then filter through absorbent cotton and paper until clear. Store in flasks as stock broth after autoclaving at 15 pounds' pressure for one-half hour.

This stock can be diluted for use as desired, according to Hottinger ten, twenty or more times. Diluted one-half (1 part stock broth, 1 part water) this broth gives excellent results with the ordinary laboratory organisms. The diphtheria bacillus, however, does not grow well.

Hottinger Agar.—Add sufficient water to Hottinger stock broth to make the required dilution. Add agar 1.5 per cent. and proceed as usual.

This agar made with Hottinger broth, 1 to 1 dilution, gives good results in successive slant agar cultures of the usual laboratory organisms with the exception of the gonococcus and the diphtheria bacillus.

Broth Prepared with Trypsin.—Use 300 to 500 grams of meat to 1 liter of water to which 0.4 per cent. sodium carbonate has been added. Soak overnight, then heat to 80° C. Cool to 38° C. and add 15 c.c. of liquid trypsin. Keep at 38° C. for five hours, stirring frequently. If kept overnight at this temperature, add toluol 10 c.c. (or thymol crystals). Then add hydrochloric acid (normal) to neutralize. Boil seven minutes, strain, set reaction. Boil one-half hour, filter and sterilize.

Peptic Digest Broth of Liver, Beef or Human Placenta.[1]—1. Wash, clean and mince 5 or more large pigs' stomachs. Mince an equal amount of clean pigs' or beef liver, cheap fat-free beef, placenta or blood clots.

2. Mix in the following proportions: Minced stomachs, 400 grams; minced liver, beef, placenta, 400 grams; HCl, 40 grams; tap water at 50° C., 4000 grams; and keep mixture at 50° C. for eighteen to twenty-four hours. (Use glass or porcelain receptacles, *e. g.*, museum jars in electrically regulated water bath).

3. Make biuret[2] and tryptophan[3] tests.

4. Transfer to large bottle and steam for ten minutes at 100° C. to stop digestion. Strain digest through cotton or preferably store overnight in ice-box and decant after twenty-four hours.

5. Warm the filtrate, or decanted digest, to 70° C. and neutralize with sodium carbonate (twice N/1 Sol.) to litmus at this temperature.

6. Sterilize (if not to be used at once) in the autoclave at 10 pounds' pressure for fifteen minutes or for thirty minutes at 100° C. on two successive days and store away.

Stock Digest may be used for: Plain digest broth; sugar-free digest broth; stock digest agar; tryptonized digest broth; sugar-free tryptonized digest broth.

The liver digest agar is especially satisfactory as a substitute basis for the colon-typhoid media which are discussed on p. 129.

Dilutions of Media.—Berry[4] has found that good results are obtained in milk plates with a medium containing the usual amount of agar and lessened amounts of meat extract, peptone and salt. With the progressive dilution of the medium there is, at first, an increase in the number of colonies, but beyond a certain point they tend to become smaller and less distinct. On agar made with a dilution of one-twelfth the usual amount of meat extract, peptone and salt, the colonies are practically identical in number and appearance with those on the standard agar.

[1] Stichel and Meyer: Jour. Infect. Dis., 1918, **23**, 78.

[2] To 5 c c. of filtered digest, add 0.1 c.c. 5 per cent. solution $CuSO_4$; mix and then add 5 c.c. N/1 NaOH. Pink color indicates complete peptonization.

[3] To 10 c.c. neutralized and filtered digest add slowly bromin water until the maximum purple coloration is reached.

[4] Coll. Studies, Dept. Health, City of New York, 1913–1915, **8**, 288.

Still better results for milk work are given by agar made with a 1 to 15 dilution of Hottinger stock broth (p. 120).

Used Agar.—It has been found by Berry that veal agar which had been used for preparation of antigens and vaccines in large quantities, could be used a second time for culture purposes. After the surface growth is removed by scraping and washing with salt solution or distilled water, the agar is melted and the different lots poured together. The reaction is then adjusted and the agar tubed and sterilized.

Except for a loss of transparency, which could probably be restored by fresh clearing and filtering, the previous use of the agar seems in no way to affect its value as a culture medium. Typhoid, paratyphoid, coli, staphylococcus, streptococcus, pneumococcus, gonococcus, diphtheria and other varieties of ordinary laboratory organisms grow as vigorously as on new agar, and in some cases the growth is heavier.

Media with Chief Ingredient other than Meat.—Peptone Solution (Dunham's).[1]—Water 1000 c.c., peptone 10 grams and sodium chloride 5 grams. Dissolve by heating and filter through filter paper. Reaction should be about pH 7.

Milk.—The milk used must be unpasteurized and as fresh as possible, preferably the best grade obtainable. Steam the milk in the Arnold sterilizer for a half-hour and place on ice for several hours or overnight to allow the cream to rise. By means of a siphon remove the milk from below the cream layer. The milk may be tubed or litmus solution added (*litmus milk*). If a good grade of milk is used no change of reaction is necessary; if acid, sufficient sodium hydrate solution should be added to render it slightly alkaline to litmus; if very acid, the milk should be discarded.

Whey Broth.—Milk (unpasteurized), 3 quarts. Let stand overnight and take off cream in morning. Heat milk on stove and stir until ready to boil. Add 6 c.c. glacial acetic acid (0.2 per cent.) to coagulate the casein. Strain through cheesecloth. Add peptone, 2 per cent.; sodium chloride, 0.5 per cent. Boil, stirring to dissolve peptone and salt. Titrate and adjust to 0.5 per cent. acid to phenolphthalein. Add white of 3 eggs to clear after cooling to 45° C. Filter through cotton and paper. Sterilize in autoclave at 15 pounds for thirty minutes.

Nitrate Broth.—Water 1000 c.c., peptone 1 gram, and nitrite-free potassium nitrate 0.2 gram.

Egg Media.—Egg media are usually coagulated and therefore solid. Use fresh-laid eggs, as they are free from bacteria.

Plain Egg (*Dorset*[2]).—The eggs are thoroughly cleansed with water of any adherent dirt, washed with 5 per cent. carbolic solution and allowed to dry partially. The ends are then gently dried in the flame and pierced with sharp forceps which have been flamed. The hole at one end should be about $\frac{3}{8}$ inch in diameter and the membrane broken; at the other end which is to be blown into, the hole should be smaller and the membrane left unbroken, if possible. The eggs are blown into a sterile Erlenmeyer flask, the blowing being done from the cheeks. To the egg is added water (10 per cent. by volume of the weight of the eggs). This is mixed by twirling the flask or by gently stirring with a glass rod. Bubbling must be avoided. The mixture is filtered through cheesecloth by gravity and tubed. (See Apparatus, Fig. 43.) The tubes are slanted and coagulated by heating to 70° C. for two or two and a quarter hours on two successive days. No further sterilization is employed. The medium is incubated to test its sterility.

Glycerin Egg (Lubenau).[3]—Ten eggs are blown into a flask and 200 c.c. of glycerin broth (5 per cent. glycerin, neutral or slightly alkaline to litmus, (about pH 6.8 to 7) added. The further preparation is the same as the preceding.

If the medium requires it, a drop or two of water can be added after coagula-

[1] Am. Jour. Med. Sci., 1893, **105**, 73.
[2] Am. Med., 1902, **3**, 555.
[3] Hyg. Rundschau, 1907, **17**, 1455.

tion to supply the necessary moisture. To conserve the moisture the plugs should be paraffined or cut off and burned and a charred cork used to seal the tube. (See also Anilin Dyes in Media.)

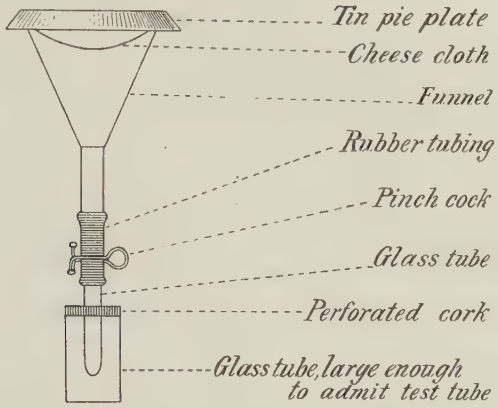


FIG. 43.—Straining and filling apparatus. The lower tube is plugged with cotton and the top wrapped. It is necessary to loosen the pinch-cock before heating. The whole is sterilized in the autoclave. (Krumwiede after Maassen.)

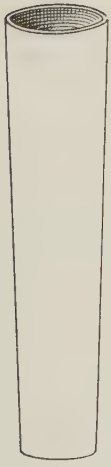


FIG. 44.—Potato borer.

FIG. 45.—Cylinder of potato, line of cut.

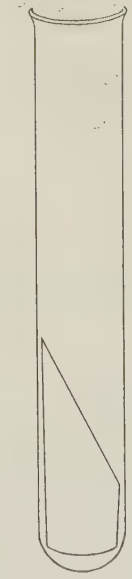


FIG. 46.—Finished medium.

Potato Media.—Use large white potatoes and scrub them thoroughly under running water. Cylinders are then cut by means of an apple-corer (Fig. 44). Cut the skin off the ends of the potato cylinder and by an oblique cut make

wedge-shaped pieces (Fig. 45). A good butt should be left or the pieces will curl on sterilization; curling occurs also if the pieces are too small and thin. While the potatoes are being prepared they should be kept under water to avoid discoloration of the cut surfaces. The reaction of potatoes is acid. This must be corrected by soaking them either in running water overnight or in a 1 to 1000 solution of sodium carbonate for several hours. The pieces are then placed in test-tubes, one inch or more in diameter (Fig. 46); a little water may be added to avoid drying. Sterilize in the autoclave at 15 pounds' pressure for thirty minutes.

Glycerin Potato for the cultivation of tubercle bacilli. Potatoes should be cut as above, soaked overnight in 1 to 1000 sodium carbonate solution, drained, and covered with a 5 per cent. glycerin solution for twenty-four hours. When tubed, the glycerin solution or water is added for maintaining the moisture.

Potato Juice Media.—**Potato Juice.**—White potatoes, grated (or run through chopping machine), 1 pound to 1 liter of water. Soak overnight. Heat to boiling. Press through cheese-cloth. Add one egg per liter. Autoclave one-half hour to clear. Filter through cotton (very tedious). Store in flasks and sterilize in autoclave one-half hour at 15 pounds' pressure.

Agar.—To veal agar add 5 per cent. potato juice and 5 per cent. glycerin.

Broth.—Meat infusion and potato juice are mixed in equal parts. Peptone and salt are added as in ordinary bouillon.

Bordet-Gengou.[1]—Water 1000 c.c., glycerin 80 c.c., potatoes (sliced) 500 grams. Heat in an autoclave at 15 pounds' pressure for one-half hour. Pour off the liquid. To 500 c.c. of this potato extract add salt solution (0.6 per cent.) 1500 c.c., and agar 60 grams. This makes a 3 per cent. agar. Autoclave to dissolve, filter and tube. Sterilize in autoclave for one-half hour at 15 pounds. When used, an equal quantity of defibrinated blood is added. (More often, 1 part of blood to 4 parts of agar). Povitzky[2] has found that a more acid medium (pH 6.6) is better for the isolation of the Bordet-Gengou bacillus. Such a medium at the same time inhibits the growth of the influenza bacillus.

Synthetic Media.—For certain work it is an advantage to know the exact chemical constitution of the medium. Then, too, some bacteria, especially certain species in soil, refuse to grow on the more complex media. Pigment production is very easily observed on these media.

Uschinsky's (Fränkel's Modification).[3]—Water 1000 c.c., asparagin 4 grams, ammonium lactate 6 grams, Na_2HPO_4 2 grams, NaCl 5 grams.

Modified Formula for Disinfection Tests.—Water 1000 c.c., asparagin 6 grams, sodium phosphate (ortho) 2 grams, sodium chloride 5 grams. Dissolve and if necessary render alkaline to litmus by addition of NaOH. Sterilize in small tubes and test for color production with *B. pyocyaneus*.

Ringer's Solution.—Sodium chloride 10 grams, potassium chloride 0.2 gram, calcium chloride 0.2 gram, sodium bicarbonate 0.1 gram, glucose 1 gram, water 1000 c.c.

For broth add 1 or 2 per cent. peptone.

For agar add 1.5 or 2 per cent. agar and 1 or 2 per cent. peptone.

Dissolve, clear, and filter.

Locke's Solution.—To isotonic salt solution (0.9 to 1.0% sodium chloride) add 0.01 per cent. chloride of potassium and 0.02 per cent. chloride of calcium. This gives an "indifferent" solution, that is, one in which tissues neither swell nor shrink; in this respect it resembles the body fluids in that it prevents osmosis.

Special Additions to Media —Carbohydrates.—These are added to media for two purposes: (1) to determine the presence of a *fermentative action* of the inoculated organisms with consequent production of acid and gas; (2) for *enrichment*, that is, as an added foodstuff to cause a more abundant growth. Carbohydrates may be used in any medium, either fluid or solid. When meat extract or meat

[1] Ann. de l'Inst. Pasteur, 1906, **20**, 731.
[2] Jour. Infect. Dis., 1923, **32**, 1.
[3] Hyg. Rundschau, 1894, **4**, 769.

infusion is used as a basis it must be remembered that both contain fermentable muscle sugar. If such a medium is prepared to determine whether a certain sugar is acted upon or not the muscle sugar must first be removed. This is done as follows:

Preparation of Sugar-free Media.—Adjust the reaction of the meat extract or infusion to about pH 7 (or slightly alkaline to litmus). To each liter add a broth culture of B. coli or one of its allies. Incubate for forty-eight hours. The bacillus ferments the sugar present. The infusion is then sterilized in the autoclave. Test again for production of acid or gas before using as a basic medium for addition of various carbohydrates. Serum water (Hiss, p. 126) may be used also as a basis.

Add carbohydrates to sugar-free media (see above), using only the purest carbohydrates obtainable. The following is a partial list of substances which may be employed. These are added usually in 1 per cent. amounts with the exception of glycerin; of this 5 per cent. is often used.

Mono-hexoses—dextrose, levulose, galactose and mannose.

Bi-hexoses—saccharose (sucrose), maltose and lactose.

Tri-hexoses—raffinose.

Polyhydric-alcohols—erythrite (tetra), adonite (penta) and mannite, sorbite, dulcite, and glycerin (hexa).

Pentoses—arabinose, xylose and rhamnose.

Polysaccharids—glycogen, dextrin and inulin.

Besides the true carbohydrates, various glucosides, such as salicin, coniferin, etc., are used, as well as carbohydrate-like substances, like inosite.

Many of the carbohydrates are very susceptible to heat. They are split into simpler compounds which may be fermented by an organism unable to ferment the unaltered sugar and thus lead to error. The usual method is to add sufficient sugar to sterile water to make a 10 per cent. or 20 per cent. solution and heat this in small containers in the Arnold sterilizer for one-half hour on three successive days. This solution is then added under sterile conditions to the sterile medium in sufficient amounts to give a final 1 per cent. content.

Certain sugars, especially some samples of maltose, are so easily split by heat that they must be sterilized by filtration.

In routine work with glucose, lactose, saccharose, mannite, and dulcite it is usually sufficient to add the sugar to the media and sterilize by intermittent sterilization. Although there may be a slight destruction of the glucose, it is not enough to be important. Inulin is an exception. Because of the resistant spores commonly present, the inulin solution should be sterilized in the autoclave.

Fluid-sugar media, with the exception of serum-water media, are usually filled into fermentation tubes (p. 95), when gas production is to be determined.

Addition of Enriching Substances.—Serum, blood, albuminous body fluids, or tissue extracts may be added to media to give additional substances for producing growth.[1] This use of carbohydrates has already been given (see above).

Under aseptic conditions one part of the enriching substance is added usually to two or three parts of the medium. If additions are made to melted agar it must be cooled to 45° C.

When slants are made, they should be allowed to set thoroughly, preferably overnight, before raising them, or they may slip down. For certain work it may be necessary to use a 2 per cent. agar to get sufficient stiffness so that the surface is not easily broken; this holds especially with media for plating where surface inoculation is done. Media thus prepared are spoken of as blood agar or broth, ascitic agar or broth, serum agar or broth, etc.

Serum Media.—For special purposes serum or other albuminous fluids, such as ascitic fluid, are used. They may be used as such or diluted with saline or by the addition of nutrient media. For these special purposes, sterilization by heat or even filtration should be avoided.

[1] See Rettger, L. F., for investigations concerning group factors, Jour. Bacteriol., 1918, **3**, 103.

Serum-water Media. — If serum is dialyzed to remove the salts, it can be heated to 100° C. without coagulation. Serum diluted with two or three times its volume of distilled water can be sterilized in the Arnold sterilizer. This is so-called "serum-water medium" (Hiss[1]) to which 1 per cent. of sugar is added for fermentation tests. Acid production is shown by the change in the indicator added and by coagulation of the serum. Gas production is shown by the bubbles in the coagulum. This medium may fail to give positive fermentation results. If peptone is added to the medium after sterilization the results are more reliable. Sufficient of a sterilized 10 per cent. solution of peptone may be added to give a final contents of about 1 per cent.

Inulin serum-water medium is the most commonly used preparation of this type. (See Carbohydrates.)

A similar medium is prepared as follows: Inulin (or other carbohydrate) 4 grams, peptone 4 grams, water, 200 c.c. Add litmus or other indicator. Dissolve and tube (2 or 3 c.c. to a tube) and sterilize, the method depending on the sugar used and precautions already given. After cooling add to each tube an equal quantity of sterile ascitic fluid or serum.

Coagulated Serum for Production of Solid Media.—Beef blood may be obtained at the slaughter house where it is placed in tall cylindrical vessels which are sterile. These should not be disturbed until the coagulation is complete. If the coagulum adheres to the sides of the vessel, it should be loosened with a sterile glass rod. After twenty-four hours on ice the serum is pipetted or syphoned off. If bloody, it can be placed on ice to allow the corpuscles to settle out. Dissolved hemoglobin is not desirable.

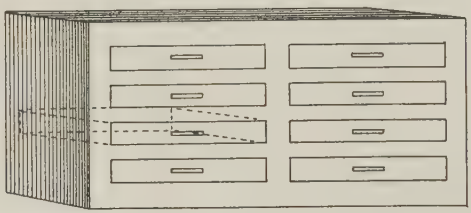


Fig. 47.—Serum inspissator (Park). Ringstands are used for support, and the tilt is easily varied as needed.

Löffler's Blood Serum.—Mix 3 parts of beef or sheep serum and 1 part of nutrient broth (neutral to litmus, about pH 6.8 to 7) to which 1 per cent. of dextrose has been added. The mixture is run into tubes and the tubes slanted in an apparatus where the temperature can be slowly raised to between 80° and 90° C. The heating in any case should not exceed 95° C. until the medium is coagulated, or bubbling will occur and the surface will be spoiled. A Koch serum coagulator may be used for this purpose or a small water-oven or even an Arnold sterilizer, covering the top with a cloth instead of the usual cover. A convenient inspissator we have devised is shown (Fig. 47). It has the advantage that the temperature can be controlled and each tube heated equally. After coagulation the tubes should be sterilized in the Arnold sterilizer for twenty minutes on three successive days. The medium may be coagulated and sterilized in the autoclave by allowing the temperature to rise very slowly until 110° C. is reached. This is rapid and convenient. This medium is especially useful for the isolation of the diphtheria bacillus. Williams has found that an equally good medium for this purpose is vitamin broth (see p. 128) base plus 0.5 per cent. dextrose, to which is added 3 parts of horse serum or plasma. Then proceed as above.

Coagulated Blood-serum Media.—Serum from the cow, horse, sheep, or dog may be coagulated in the form of slants. This type of medium has been used with and without the addition of glycerin for cultivation of tubercle bacilli,

[1] Jour. Exper. Med., 1905, **6**, 317

but a more satisfactory medium can be prepared from eggs (p. 122). The serum used should be sterile and, after tubing, heated to 70° C. for one hour. If a clear medium is desired, the best way is to remove a tube from time to time for observation and cease heating when set sufficiently. Higher temperatures or too long an exposure render serum opaque.

Serum may be coagulated in high columns in test-tubes especially for cultivation of spirochetes. The tubes should be warmed to 65° C. and a few at a time placed in water at 75° C. These are tilted every few seconds and removed as soon as they start to set. The heat retained in the tube will complete the coagulation. In this way a soft, almost transparent, coagulum is formed.

The serum may be diluted with saline, broth, etc., for special purposes and treated in the same way.

Blood Media (Hemoglobin).—Blood may be added to media in the form of whole blood, defibrinated blood or citrated blood. It must be obtained under aseptic conditions, as it cannot be sterilized. From the larger animals it is most easily obtained by introducing a sterile trocar into the jugular vein after carbolizing the site. The carbolic solution must be washed off with alcohol, which should be allowed to dry. With smaller animals anesthesia is used and the carotid artery is dissected out for the introduction of a trocar or cannula. Small quantities of blood may be obtained by puncturing the heart with a sterile syringe.

The fluid blood may be mixed at once with the medium. If the blood is to be stored it is either defibrinated by shaking it in the container, which contains sterile glass beads or spiral pieces of wire, until the fibrin has formed, or it is mixed with sodium citrate solution. One part of 10 per cent. sodium citrate solution is added to nine parts of blood.

Blood may be added to either fluid or solid media in the proportions given on page 125. Such media are opaque. Blood heated at a higher temperature than 45° C. makes a better culture medium for certain organisms. This is done by adding the blood to agar at 95° C., which produces the so-called **chocolate agar.** A final dilution of the unheated blood 1 to 500 gives a good growth with the influenza bacillus, though usually a dilution of 1 to 30 is used.

Blood-drop Agar.—A drop of blood may be added to the surface of solid agar, forming "blood-drop agar." This may be in the form of slants or plates. When these are inoculated the blood is streaked over the surface, together with the culture, by means of the platinum loop.

The chocolate and blood-drop agar are especially good for the influenza bacillus. The streptococcus and its allies grow well in all blood media.

Tissues in Media.—Watery extracts of tissues, especially of placenta, are used in the same manner as sera. Add 500 grams of ground-up tissue to 1 liter of water and extract as for meat infusion but apply no heat. After a preliminary filtration through paper or sand, the fluid is sterilized by filtration through a filter candle (p. 117).

Use of Fresh Tissue in Media.—Sterile fresh tissue may be added to media. This not only adds nutrient material but aids the production of anaërobic conditions. Smith[1] first used it in fermentation tubes for growing anaërobes, Williams[2] used it on agar for the growth of pure cultures of amœbæ, and Noguchi[3] uses it in special media for spirochetes and other feebly growing organisms. In the latter medium rabbit's kidney is most commonly employed. Small pieces are added to fluid media or placed in test-tubes and serum or ascitic agar added. In the case of coagulated serum the tissue is pushed to the bottom of the tube.

Media Heated after the Addition of Tissues, etc.—Especially good for the pathogenic (proteolytic) anaërobes. Drop a piece of meat into gelatin or broth tubes and sterilize; or cook the meat in water, alkalinize, sterilize and tube, *e. g.*, bullock's heart minced finely and then ground in a mortar, 8 ounces to tap-water 8 ounces.

[1] Jour. Med. Res., 1905, **14**, 196.
[2] Ibid., 1911, **20**, 263.
[3] Jour. Exp. Med., 1912, **16**, 199.

Egg protein may also be used. Add 1 egg to 300 c.c. of water, mix, and bring to boil slowly, shaking frequently, tube and sterilize. An alkaline-egg mixture, yolk of 1 egg and whites of 2, to 500 c.c. of water and 6 c.c. of N/1 NaOH may be used (as under Cholera Media) in proportion of 1 to 5 in broth or agar. (Robertson.)[1] See also milk, and inspissated serum or egg media for other specially suitable media.

Potato Juice (p. 124).—Potato juice may be added either alone or with glycerin. To veal agar add 5 per cent. potato juice and 5 per cent. glycerin. This is glycerin potato agar.

Potato Broth.— Meat infusion and potato juice are mixed in equal parts. Peptone and salt as in ordinary broth.

Nutrose.—May be used in media in 1 per cent. amounts to aid growth. The difficulty of obtaining nutrose during the war led R. L. McKenzie Wallis[2] to investigate its real composition and to suggest the use of a substitute nutrose prepared as follows: Peanut flour 94 parts, casein 5 parts, and sodium carbonate 1 part are mixed to form a powder. This is used as the powdered nutrose. The peanut flour and casein are insoluble in water unless the sodium carbonate is present.

This substitute is apparently helpful, but judging from some experiments made with the Conradi-Drigalsky medium prepared without any nutrose at all, it would seem to be not really necessary.

Retention of Special Substances.—Vitamin Agar.—In this agar special nutritive substances, "vitamins" are retained by the process of preparation. Lloyd[3] has shown that in the contact of media with cotton and paper, for the purpose of filtration, the vitamins are removed. This may be prevented by the use of sedimentation instead of filtration (Williams) or by the use of the Sharples centrifuge (Huntoon).

To 500 grams of fresh beef heart, trimmed free from fat and tendon, add 500 c.c. of tap water, peptone 15 grams, sodium chloride 5 grams and 1 egg well beaten. Heat this mixture (A) in a water-bath, double boiler or over the open flame, with constant stirring, until the color changes to brown (at about 68° to 70° C.). Strain through a fine wire sieve or wire gauze. Do not use cheesecloth, cotton or paper.

Dissolve 15 grams of agar in 500 c.c. of tap water, mixture (B). Cool this mixture to 70° C. and add to (A). Set the reaction either to +0.2 phenolphthalein or to about pH 7.4. Heat the mixture in an autoclave at 15 pounds' pressure for thirty minutes. Remove the kettle carefully and set aside for sedimentation (p. 113) to take place, or run in Sharples centrifuge. If the sedimentation method is used, turn the solid agar out of the kettle in mould form and trim off the layer of sediment and discard it. The remainder is fairly clear.

Melt, tube and sterilize in an autoclave, at fifteen pounds, for thirty minutes.

This medium is especially useful for the growth of meningococcus. It is recommended as a base for chocolate medium and other blood media.

Vitamin broth may be made in the same way.

Use of Insoluble Carbonates.—The value of the carbonates in media lies in the fact that they neutralize the acids produced by bacterial growth. These acids, produced especially when sugars are present, tend to prevent further growth or even injure the viability of the culture. The exact manner in which the carbonates act is not known. They are added chiefly to fluid media either in the form of calcium carbonate powder or of crushed marble. About 1 per cent. by bulk is sufficient. The marble must be white, not streaked with pink or brown. It is broken into pieces the size of a pea or smaller, washed in running water and sterilized in the dry air sterilizer before being added to the medium. Sterilize the medium in the autoclave at 15 pounds' pressure on two successive days as resistant spores are often present.

[1] Jour. Path. and Bact., 1916, **20**, 327.
[2] Indian Jour. Med. Res., 1917, **4**, 786.
[3] Jour. Path. and Bacteriol., 1916, **21**, 113.

Broth prepared with these additions is known as *carbonate* or *calcium* broth. It is useful especially for the streptococcus-pneumococcus group. When the medium is used in large amounts the containers should be shaken from time to time.

Addition of Inhibiting Substances.—Anilin Dyes.—Various basic anilin dyes show a differential restraining action on the growth of bacteria. Gentian violet (and allied dyes) when present in a dilution of 1 to 100,000 preferably in agar, inhibits the growth of the Gram-positive group of bacteria but has no effect on the growth of the Gram-negative bacteria. This is a general rule. It may be made use of to prevent growth of Gram-positive germs or for isolation of Gram-negative germs from contaminated material where the contamination is Gram-positive. An example of this is the use of crystal violet in Conradi medium. Exceptions to the above general rule occur, thus the acid-fast but Gram-positive group grow freely even when gentian violet in strong concentrations is used. Because of this, pure cultures of acid-fast bacilli (B. tuberculosis) can be obtained by this means from contaminated material, as sputum or feces.

Gentian-violet-egg Medium (Petroff).[1]—Meat extract (veal or beef 500 grams to 500 c.c. of 15 per cent. glycerin in water, extract for twenty-four hours and collect fluid by means of a press; sterilize by filtration). Add 1 c.c. of a 1 per cent. alcoholic solution of gentian violet to each 100 c.c. of meat extract, mix with 2 parts of whole egg (p. 122), tube and inspissate on three days for three-quarters of an hour at 80° to 85° C. This medium is especially recommended for the isolation of B. tuberculosis.

Crystal Violet Potato Medium (Corper[2]).—This is prepared by cutting large cleaned pealed potatoes free from surface defects into cylinders about 3 inches long and $\frac{5}{8}$ inch in diameter. The cylinders are halved longitudinally and immediately soaked in 1 per cent. sodium carbonate solution containing 73,000, or 0.0015 per cent, crystal violet (the dye and sodium carbonate solution should be mixed just before using to prevent decolorizing) for one to two hours. They are then gently wiped with a clean towel, introduced into sterile culture tubes ($\frac{3}{4}$ by 6 inches) containing 1.5 c.c. of 5 per cent. glycerin broth, plugged and sterilized in the autoclave at 15 pounds' pressure for at least one-half hour. Before inoculating the material must be emulsified, and inoculated in 6 per cent sulphuric acid for thirty minutes, then diluted with saline, centrifuged and the residue inoculated.

Special Media for Typhoid, Paratyphoid, Dysentery and Colon.—Certain dyes have a differential action on closely allied bacteria inhibiting completely or partially the growth of one, but allowing another to grow freely. An example of this is the action of brilliant green on the typhoid, paratyphoid and colon bacillus. The colon types are completely or almost completely restrained, typhoid less so and paratyphoid least of all. This is applied in isolation of typhoid or paratyphoid from stools. (See below.) The use of dyes as indicators has been already noted (p. 109). Numerous media are used. The most commonly employed plating media are Endo and Conradi-Drigalsky.

Endo Medium (Kendall's Modification).[3] — The basis is ordinary beef extract agar, slightly alkaline to litmus or about pH 7. This should be sterilized in bottles in 100 c.c. amounts. When needed, 1 gram of lactose is poured into a bottle of agar and the agar melted, which dissolves and sterilizes the lactose. To each bottle, after melting, is added 1 c.c. of decolorized fuchsin prepared as follows: To 10 c.c. of a freshly prepared 10 per cent. watery solution of sodium sulphite add 1 c.c. of a saturated alcoholic solution of fuchsin and heat in the Arnold sterilizer for twenty minutes. Plates are then poured and allowed to harden without the covers, and dried in the incubator for thirty minutes, protecting the plates from dust. The medium must be mixed each time it is needed and the plates used fresh for the color returns gradually and the plates are then useless. (See

[1] Jour. Exper. Med., 1915, **21**, 38.
[2] Jour. Am. Med. Assn., 1928, **6**, 371.
[3] Jour. Med. Res., 1927, **25**, 95.

also Media for Water Examinations.) Kligler and Defandorfer recommend a reaction of pH 7.6 to 7.8 for the basic agar, the substitution of the bisulphite salt and the addition of 0.5 c.c. of the fuchsin sulphite mixture, for the B. dysenteriæ group.

Robinson and Rettger's Modification of Endo's Medium.[1]—This medium has given sharper differentiation than the above medium.

Preparation of agar: Water, 1000 c.c.; agar, 25 grams; peptone (Fairchild's), 10 grams; meat extract, 5 grams. Dissolve the agar, meat extract, and peptone. Make neutral to litmus (about pH 6.8), autoclave for thirty minutes at 15 pounds' pressure. Filter through cotton and cheese-cloth. Add 10 c.c. of a 10 per cent. sodium carbonate solution; heat for ten minutes; add 1 per cent. lactose, 5 c.c. saturated alcoholic fuchsin, and finally 10 c.c. of a 10 per cent. solution of anhydrous sodium bisulphite. The medium is now tubed in 20 c.c. amounts and autoclaved for seven minutes at 10 pounds' pressure. The best results are obtained when the reaction of the medium is brought, after the addition of the lactose and sodium bisulphite, before adding the fuchsin, to +0.1 phenolphthalein, hot titration.

According to our experience it seems better to prepare the medium, adjust the reaction to 0.4 (about −pH 8.6 or 8.8). The sterilization and the subsequent addition of the bisulphite brings the reaction to the desired end-point, pH 8.2; bottle in 100 c.c. amounts, and add the lactose, fuchsin, and sodium bisulphite just before use.

Methylene-blue Eosin Agar (Holt-Harris and Teague).[2]—Basis, extract agar of reaction of +0.8. Add lactose and saccharose, 0.5 per cent. and then to each 50 c.c. of agar, 1 c.c. of a 2 per cent. yellowish eosin solution and 1 c.c. of a 0.5 per cent. methylene-blue solution. Shake and pour plates. The agar acts as a protective colloid preventing the mutual precipitation of the eosin and methylene blue. This protection is destroyed when acid is produced in the colonies, which then show as black or show black centers. If the plates are too crowded this change may not develop. The saccharose is omitted when used for dysentery isolation. B dysenteriæ (Shiga) may fail to grow on this medium.

Conradi-Drigalsky Medium.[3]—Water 1000 c.c., agar 20 grams, sodium chloride 5 grams, peptone 20 grams, nutrose 10 grams, beef extract (Liebig's) 4 grams, normal NaOH 50 c.c. Dissolve the ingredients in an autoclave, cool, and clear with eggs. Adjust reaction to a moderate but distinct alkalinity to litmus. To each liter of agar thus prepared are added 130 c.c. of Kubel and Thiemann litmus solution, crystal violet (1 to 1000 solution) 10 c.c., and 15 grams of lactose. Heat in an Arnold sterilizer ten minutes to obtain thorough mixing and fill in tubes or bottles and sterilize in Arnold sterilizer. Omit the crystal violet if to be used for dysentery.

Russell's Double Sugar Medium.[4]—To ordinary beef extract agar adjusted neutral to litmus add 1 per cent. of lactose and 0.1 per cent. of glucose and sufficient litmus to give a good color. Tube and slant, leaving a generous "butt" at bottom of tube for stab inoculation. Krumwiede[5] has modified this medium by substituting 1 per cent. of the Andrade indicator and adding 1 per cent. of saccharose. This gives sharper color changes and the fermentation of saccharose excludes the paratyphoid-like "intermediates" frequently found in stools.

Brilliant Green Agar (Krumwiede[6]) for Isolation of B. Typhosus and B. Paratyphosus.—The basis of the medium is prepared as follows:

A. Water 1 liter, agar 30 grams; autoclave into solution.

B. Water 1 liter, meat extract 6 grams, salt 10 grams, peptone 20 grams; Arnold until dissolved.

[1] Jour. Med. Res., 1916, **29**, 363.
[2] Jour. Inf. Dis., June 6, 1916, vol. **18**.
[3] Ztschr. f. Hyg., 1902, **39**, 283.
[4] Jour. Med. Res., 1911. **20**. 217.
[5] Jour. Med. Res., 1917, **32**, 225.
[6] Jour. Infect. Dis., 1916, **18**, 1 and 1918, **33**, 275.

Mix A and B; add normal soda (see below); boil one-half hour. Cool, clear with egg, filter until clear, bottle and autoclave. The agar, when used, must be neutral to Andrade's indicator or pH 6.9 (p. 110). We find it convenient, on account of the necessity of standardizing the agar, to prepare large batches (20 liters); if this is done, the entire bulk of agar intended for use in brilliant green medium must be well mixed in one container after filtering. At the time the medium is to be used, a number of bottles are melted, and to each 100 c.c. is added 1 c.c. of indicator (Andrade's), 5 c.c. of a sterile solution in distilled water of 20 per cent. of lactose and 2 per cent. of glucose (this will give 1 per cent. lactose and 0.1 per cent. glucose in the agar), and finally the appropriate amount (see below) of a 0.1 per cent. solution of brilliant green in distilled water. The medium is well mixed and poured into rather thick plates (no more than six from 100 c.c.). Porous tops are convenient, as dry plates are essential.

The selective bactericidal action of brilliant green is exhibited only at certain high dilutions. It is quantitative and varies according to the medium and the material inoculated. Even with similarly prepared batches of agar, different amounts of dye may be required to obtain the same restraining action. It is therefore necessary to determine for each batch the optimal dilutions for use, and these dilutions and the same dye solution are used for the rest of that preparation.

The method of standardization is to pour plates of four dilutions of dye: 1 to 500,000, 1 to 330,000, 1 to 250,000, 1 to 200,000, which correspond to 0.2, 0.3, 0.4, 0.5 c.c. of 0.1 per cent. dye solution to 100 c.c. of agar. These plates are evenly inoculated with broth cultures of freshly isolated strains of B. typhosus which have been diluted to give 75 to 200 organisms per loopful (loop of broth culture to 15 c.c. of broth), and with dilutions of broth cultures in suspensions of normal stools. This gives fairly satisfactory results. The best material for standardization is positive typhoid stools. A plate of each dilution of dye and a control plate of a non-restraining medium, such as Endo's, are used for each culture or stool.

The appropriate dilutions are (1) the lowest dilution at which the typhoid colonies are of good size and undiminished in number as compared with the control plate, but many fecal types are excluded; (2) a lower dilution, where the typhoid colonies are slightly reduced in size and number, but almost all the other flora have disappeared, the degree varying with different stools. They correspond, in general, to the effect we obtain with 0.2 and 0.3 c.c. respectively. For paratyphoid, the dye can usually be safely used in lower dilutions: 1 to 200,000. In investigating an outbreak of paratyphoid the medium can be standardized directly against the offending strain as soon as one is isolated. We have found two dilutions preferable for routine examinations, as the fecal proteins, which vary in amount in different suspensions, reduce somewhat the activity of the dye; and, moreover, the fecal flora of different stools as well as various strains of typhoid vary in their dye sensitiveness. With two concentrations these variables are balanced. For an occasional examination with unstandardized materials, 0.2, 0.3, 0.4 (0.5 if paratyphoid is suspected) will probably cover the range of variation.

To inoculate, the stool is emulsified (if fluid, it may be slightly diluted) in about 15 volumes of saline or peptone solution and the heavier particles are allowed to sediment for one-half hour. For routine work, 2 strong dye, 2 weak dye and 2 Endo plates are used for each sample; 1 loopful of suspension is placed on a weak dye plate, the same on a strong plate; these are evenly smeared with a heavy platinum or nichrome spreader, which is then carried over to an Endo plate which has received no direct material; a second series of weak and strong dye plates is inoculated with twice this amount, and spread in the same way to an Endo plate. The spatulum is not flamed between plates. This will usually give satisfactory seeding of Endo as well as dye plates, although the former receive only the material carried over on the spreader. If stronger dye is used, as for paratyphoid, more material may be inoculated.

After eighteen hours' incubation the typhoid colonies are characteristic in appearance. They are of good size (1 or 1.5 mm.), as the glucose favors their growth. Viewed through the plate against a dark background, with the light passing obliquely through the agar, they have a peculiar marking; with artificial light and hand lens, under the same conditions, they have the appearance of a coarse wool fabric. The colony may have a slight pinkish tinge from fermentation of the trace of glucose; this renders it still more distinctive. The larger colonies resemble paratyphoid bacillus; they are heavier, and more opaque, the markings are less evident, and they may be tinted a delicate green. Paratyphoid A resembles the typhoid colony more nearly than the paratyphoid B; it is frequently extremely flattened, slightly tinged, and has an ill-defined edge. All the types show better markings on stronger dye. On weak dye plates (rarely on strong) there may develop typhoid-like colonies of coliform organisms, but their exaggerated striations, with distinct crossbars, usually exclude them. Many of these typhoid-like organisms agglutinate spontaneously on the slide when the macroscopic slide technic is used for identification (see Agglutination).[1]

Digest Products for Basic Agar.—Amino-acid digests are more favorable for the growth of members of this group than are the extract-agars or even infusion media. Cole and Onslow have shown this by the acceleration of early carbohydrate fermentation in such media. Stickel and Meyer recommend especially a peptic liver digest basis (see p. 121).

Fluid Media Containing Brilliant Green.—Browning, Gilmour and Mackie,[2] Krumwiede and Pratt,[3] Torrey,[4] Robinson and Rettger[5] and many English workers have successfully used brilliant green in peptone-water or broth, sometimes with the addition of glucose, for the isolation of typhoid or paratyphoid bacilli from feces. It is essential to use graded dilutions of the dye; dilutions from 1 to 100,000 to 1 to 500,000 are generally advised. The medium is sterilized in 5 or 10 c.c. quantities in test-tubes, and the dye added just before use. After twelve to eighteen hours the highest concentration tubes showing growth are plated on Endo's medium. While the pathogenic organisms are frequently lost in this medium by overgrowth of mucoid aërogenes types, enrichment in brilliant green peptone-water or broth may give positive results where even brilliant green agar fails; for routine work, the above fluid media add too much to the time, cost and labor of examination to warrant their use except in special instances. Teague and Clurman[6] have recently reported a medium which they claim gives better results.

Special Media for Cholera.—The vibrio group will grow in the presence of a reaction so extremely alkaline that there is inhibition of the growth of most other bacteria, especially those which occur in feces.

Peptone Solution (see p. 122).

Saccharose Peptone-water (Bendick).[7]—To 1000 c.c. peptone solution, neutralized to phenolphthalein, add 1 gram of anhydrous sodium carbonate. Boil and filter. Add 5 grams of saccharose and 5 c.c. of a saturated solution of phenolphthalein in 50 per cent. alcohol. Tube and sterilize in Arnold sterilizer.

Dieudonne's Medium.[8]—Mix equal parts of defibrinated beef blood and normal sodium or potassium hydrate solution and steam in the Arnold sterilizer for a half-hour; 3 parts of this are added to 7 parts of 3 per cent. agar neutral to litmus (about pH 6.8), and poured into Petri dishes (15 c.c. to a 10 cm. dish). Let them harden uncovered, but protected by paper. Place strips of filter paper between the dish and cover to aid in the absorption of the moisture and ammonia and place in the incubator for twelve to fifteen hours. Nothing will

[1] Jour. Infect. Dis., 1918, **23**, 275.
[2] Jour. Hyg., 1913, **13**, 335.
[3] Jour. Exper. Med., 1914, **19**, 20 and 501.
[4] Jour. Infect. Dis., 1913, **13**, 263.
[5] Jour. Med. Res., 1911, **29**, 363.
[6] Ibid., 1916, **35**, 107.
[7] Centralbl. Orig., 1st Abt., 1912, **62**, 536.
[8] Centralbl. f. Bakteriol., Orig., 1909, **50**, 107.

grow on the medium when first made. The plates are good for about ten to fourteen days. Various modifications of this medium have been suggested.

PILON.—Substitute 12 per cent. sodium carbonate (crystalline) solution for the sodium hydrate. The plates can be used after thirty minutes' drying.

Alkaline-egg Medium (Krumwiede). — Make an egg-water mixture, using equal parts of egg and water. Mix equal parts of the egg-water and 12 per cent. sodium carbonate (crystalline) and steam in the Arnold sterilizer for twenty minutes. While hot mix 20 parts to 80 parts beef extract peptone (Fairchilds) 3 per cent. agar, reaction unadjusted, pour into plates and dry for twenty minutes. The colonies are increased in size if 0.2 per cent. of glucose is added. Goldberger[1] suggested the use of anhydrous sodium carbonate, and makes the egg mixture, as given, with a 6.5 per cent. solution of anhydrous sodium carbonate and mixes 1 part of the egg with 5 parts of glucose extract agar.

Sodium Oleate Agar (Avery).—This is a selective medium for the isolation of the influenza bacillus. By the action of the sodium oleate the growth of many of the Gram-positive cocci, present in nasal secretions or in the sputum, is inhibited when these materials are inoculated on this medium. It is prepared as follows:

1. A 2 per cent. nutrient agar (vitamin, p. 128, preferred) either neutral in reaction (*i. e.*, about pH 7) or slightly alkaline (pH 7.2 or 7.4). If phenolphthalein is used as the indicator make the end-reaction somewhat acid to it, that is, about +0.4.

2. Sodium oleate, neutral (Kahlbaum's preferred), a 2 per cent. stock solution in water. This may be sterilized in the autoclave.

3. Suspension of red-blood corpuscles. Sterile defibrinated human or rabbit blood may be used. Remove the corpuscles by centrifugation and discard the supernatant serum by pipetting it off. Make up the original volume by the addition of nutrient broth. Mixture: To 94 c.c. of the hot agar (90° C.) add 5 c.c. of the 2 per cent. sodium oleate solution and 1 c.c. of the red-blood cell suspension.

Medium D for the Cultivation of the Meningococcus.[2]—1. Make a double strength infusion of veal, 1 liter.

2. To 800 c.c. of distilled water add 50 grams agar, 20 grams peptone, 10 grams sodium chloride. Boil to dissolve. Add 40 grams gelatine soaked in 200 c.c. of distilled water. Combine 1 and 2, adjust to pH 7.8, clarify with egg; filter through glass wool and tube. Sterilize in the autoclave at 15 pounds pressure for thirty minutes.

Media for Special Uses other than Those Given.—The media for the detection of the typhoid-dysentery-colon group (p. 129), for the diphtheria bacillus (Chapter XIX and p. 126), for the tubercle bacillus (pp. 122 and 123), for the influenza bacillus (p. 127 and above), the pneumococcus and the streptococcus (p. 127) have been given.

Liver Agar (recommended by Dopter, Penfold and others). — Substitute minced liver for meat and proceed as in preparing infusion agar. With a reaction of +0.2 per cent. to phenolphthalein it is especially adapted for gonococcus.

Starch Medium.—(Vedder.)—Beef 500 grams, water 1000 c.c., extract in ice-chest overnight, boil and strain, add agar 1.5 per cent. and dissolve. Correct reaction to +0.2 to +0.7 per cent. phenolphthalein, *i. e.*, between pH 7 and pH 7.6. Cool and clarify with eggs. Filter, add 1 per cent. of starch and heat in Arnold for forty-five minutes, shaking the medium several times to distribute the starch. Tube and autoclave fifteen minutes at 10 pounds' pressure. Recommended especially for meningococcus.

Media for the Examination of Milk, Water,[3] **Shell-fish and Sewage.**—*Standard Methods.*—The basis for broth, agar or gelatin is 3 grams of beef extract

[1] Hyg. Lab. Bull., December, 1913, No. 91, p. 19.

[2] Eli Lilly: Direct communication.

[3] Committee on Standard Methods of Water Analysis, 1920. A. P. H. A., 92.

(Liebig's or its equivalent by tests) and 5 grams of peptone Armour's, Digestive Ferment Co., Fairchild's or their equivalent to 1 liter of distilled water. No salt is used. Gelatin media contain 10 per cent. of gelatin dried at 105° C. for one-half hour before weighing. Agar media, 1.2 per cent. agar if dried as above, or 1.5 per cent. if market agar is used. The agar should be soaked in water and washed after weighing to rid it of its salts. If the reaction of the medium does not fall between +0.5 and +1 (phenolphthalein) or about pH 6.6 to pH 7.4, correct it (see Reaction, p. 104). Usually correction is not necessary, especially with agar.

Sugar broths are prepared by the addition of 0.5 per cent. of the required carbohydrate. The reaction of sugar broth should be neutral to phenolphthalein, about pH 8. Litmus (reagent of highest purity) should be in 2 per cent. solution. Azolitmin (Kahlbaum), 1 per cent. solution, may be used as a substitute. Adjustment of reaction of the solution may be necessary. One cubic centimeter of litmus solution is added to 10 c.c. of lactose agar for litmus-lactose-agar plates when poured. All media and litmus solutions are to be tubed before sterilization and sterilized at 15 pounds' pressure for fifteen minutes, removed quickly and cooled rapidly.

The Endo recommended has as a basis an agar containing 3 per cent. of agar, 1 per cent. of peptone and 0.5 per cent. of Liebig's extract. The medium is sterilized in 100 c.c. lots. Dissolve 1 gram lactose in 15 c.c. distilled water. Dissolve 0.25 gram anhydrous sodium sulphite in 10 c.c. water. To the sulphite solution add 0 5 c.c. of a 10 per cent. solution of basic fuchsin in 95 per cent. alcohol. Add the fuchsin sulphite solution to the lactose solution and add the mixture to the melted agar just before use. For presumptive tests for B. coli lactose broth in fermentation tubes is to be used.

Milk.—Agar for milk work is prepared as follows:

Procedure A.—Dissolve the beef extract, 0.3 per cent., and peptone,[1] 0.5 per cent. in two-fifths of the total amount of distilled water by boiling on the stove. Filter this mixture through paper or paper pulp (p. 109).

Procedure B.—Agar, oven-dried, 1.2 per cent., or market agar, 1.5 per cent. Soak and wash under tap in sieve; add it to distilled water, three-fifths total amount of water minus the water absorbed by the agar during the washing. Determine this by first weighing kettle with unwashed agar and then with washed agar which has been well drained. The weight of 1 gram is considered as 1 c.c. of water absorbed.

Mix A and B (agar not yet melted). Heat mixture over stove, stirring at frequent intervals until agar is entirely melted. Then boil and stir constantly for twenty minutes; make up water lost in evaporation by adding hot distilled water. Do this by weight.

Determine the reaction (see above). Do not use eggs for clearing. Filter through cotton or paper-pulp in Buchner funnel (p. 109) or run through a Sharples centrifuge until clear. Take 10 c.c. to a tube for plates. Sterilize in autoclave for twenty minutes after the pressure reaches fifteen pounds, or in streaming steam on three successive days for twenty minutes each day after agar is completely melted.

Water.—The preparation of agar for water work differs[2] slightly from that given for milk. That the milk agar be adopted for both is to be recommended by the Committee.

Shell-fish.—Use agar as for water count. Lactose bile (see below) is still employed.

Additional Media for Water, Sewage, Shell-fish Examination.—Neutral Red Lactose Peptone.—To peptone solution add 1 per cent. of lactose and 1 per cent. of a saturated aqueous solution of neutral red. Tube in fermentation tubes.

[1] It is found by experience that with the peptone used, the addition of normal NaOH it necessary to give the required reactions. Add a suitable amount at this point. Do not add acid under any circumstances.

[2] After the agar and other ingredients are dissolved the cooling to 45° C. in a cold water bath and then warming to 55° C. in the same bath without stirring is directed.

Liver Broth.—Beef liver 500 grams, peptone 10 grams, dextrose 10 grams, dipotassium phosphate (K_2HPO_4) 1 gram, water 1000 c.c. The liver is boiled for two hours for extraction and strained. Then dissolve other ingredients and adjust reaction.

Lactose Bile.—Add 1 per cent. of peptone and 1 per cent. of lactose to ox bile. Tube in fermentation tubes and sterilize.

Brilliant Green Bile.—Add 50 grams dried oxgall and 10 grams peptone to 1 liter of distilled water and boil in double kettle for one hour. Then add 10 grams powdered lactose and 10 c.c. of a 1 per cent. solution of brilliant green; tube and autoclave for fifteen minutes under 10 pounds' pressure.

Media for Toxin Production, Etc.—Diphtheria Toxin Broth (Smith).—Lean veal (chopped), 20 pounds; water (tap), 20 liters. Place in ice-box overnight; next morning, strain. Titrate at room temperature and set reaction 1.5 per cent. acid to phenolphthalein. When chill is removed from kettle, add 10 c.c. of twenty-four hour culture B. coli for every liter and incubate at 37° C. overnight. Next morning add 1 egg for every 2 liters. Boil for twenty minutes and strain. Titrate as before and set reaction 0.5 acid (phenolphthalein). Then add: peptone,[1] 1 per cent., NaCl, 0.5 per cent. Heat to boiling to dissolve peptone and salt. Titrate and make final reaction 0.5 per cent. acid to phenolphthalein. Filter through filter paper and cotton. Put 800 c.c. in each 2-liter Erlenmeyer flask. Sterilize in autoclave at 15 pounds for one-half hour. Before inoculating flasks add to each one 8 c.c. of a 10 per cent. solution of glucose which has been sterilized in Arnold on three successive days for thirty minutes.

Diphtheria Toxin Broth (Martin's Peptone).—Lean veal free from fibrous tissue, minced, one pound to the liter of water. Incubate for eighteen to twenty-four hours at 35° C. Heat at 45° to 48° C. for one hour. Boil briskly for a half hour. Strain through cheese-cloth. Measure the filtrate and warm it to 70° C. Add an equal amount of Martin's peptone (p. 120) which has just been neutralized (about pH 7) at 70° C. A clear product usually results from mixing the two at this temperature. Heat to boiling. Titrate (room-temperature method) to +0.5 (phenolphthalein). Autoclave at about ten pounds' pressure for fifteen to twenty minutes to clear. Titrate again and adjust to +0.5 as before if necessary. Filter through cotton and paper into flasks, allowing 800 c.c. to a flask. Sterilize one-half hour at fifteen pounds' pressure.

Tetanus Toxin Broth.—One pound of lean veal per liter of water. Soak overnight in ice-chest. Heat to 45° C. for one hour, then boil for one-half hour. Strain, add peptone (Berna), 1 per cent., salt, 0.5 per cent., and glucose 1 per cent. Boil until ingredients are melted and adjust reaction to +1 per cent. Sterilize in 2-liter Erlenmeyer flasks, in Arnold sterilizer, leaving only sufficient space in flask for expansion during heating. Heat for one and a half hours on first day and for one hour on second day.

Tetanus Toxin Broth (Martin's Peptone).—Lean veal free from fibrinous tissue, minced, 1 pound to the liter of water. Soak in ice-box for eighteen to twenty-four hours. Heat to 45° to 48° C. for one hour. Boil briskly for one-half hour. Strain through cheese-cloth. Measure the filtrate and warm to 70° C. Add an equal amount of Martin's peptone which has just been neutralized at 70° C. The two liquids should be at the same temperature, for then a clear product usually results. Heat to boiling. Titrate (room-temperature method) to +1 (phenolphthalein). Autoclave at about ten pounds' pressure to clear. Measure and add 1 per cent. glucose (powder). Boil two or three minutes. Titrate again and set reaction as before to +1. Filter through cotton and paper directly into 2-liter Erlenmeyer flasks, leaving only sufficient space in flasks for expansion of broth during sterilization in the Arnold. Sterilize flasks for one and a half hours on the first day and one hour on second day.

Mallein Broth (Eye and Subcutaneous).—Lean veal free from fascia, minced, one pound to the liter of water. Macerate overnight at room temperature. Heat at 45° to 50° C. for one hour. Boil up strongly. Strain through cheese-

[1] Parke, Davis Co.: See Wilcox, Jour. Infect. Dis., 1922, **30**, 536.

cloth. Add peptone (Fairchild's) 1 per cent., sodium chloride 0.5 per cent. Boil again to dissolve the peptone. Titrate (room-temperature method) and adjust reaction to +2.5 (phenolphthalein) (about pH 6 to pH 6.4). Autoclave for fifteen minutes at fifteen pounds' pressure to clear. Filter through cotton and paper. Measure and add glycerin, 5 per cent. Put 250 c.c. broth in each quart Blake bottle or liter flask. Sterilize in autoclave one-half hour at fifteen pounds' pressure.

Tuberculin Broth.—One pound chopped lean beef per liter of water. Infuse in ice-box overnight, heat to 45° C. and hold at 45° to 55° C. for one hour. Boil up thoroughly. Strain and to the filtrate add peptone, 1 per cent., salt, 0.5 per cent. Set reaction to +1.3 phenolphthalein (pH 7.1) with normal sodium hydroxide. Boil, filtrate through paper, add 5 per cent. glycerin; put 200 to 250 c.c. into each quart Blake bottle and sterilize in autoclave at 15 pounds' for one-half hour. Final reaction +1.5 phenolphthalein (pH 6.9).

Media for Protozoa.—For Blood Flagellates (Novy and MacNeal[1]).—Equal parts of nutrient agar and fresh defibrinated blood (rabbit or rat). The medium is allowed to stiffen slanted, so more water of condensation may gather at bottom of slanted surface. The medium should be planted while fresh with blood or other infected material containing the living flagellates (*e. g.*, trypanosomes, Leishmania).

Medium for Malaria Organisms (Bass and Johns[2]).—Ten c.c. of blood drawn from a malarial patient, and carefully defibrinated. This is placed in smaller test-tubes in 1 c.c. amounts. One per cent. of a 50 per cent. solution of dextrose is added to each small test-tube before adding the blood. The red corpuscles settle so that a ½ cm. layer of serum is left about them. The parasites grow in a thin layer near the top of the cell sediments. Beneath this zone the parasites die. Bass says leukocytes should be gotten rid of because they destroy the parasites, but the Thompsons say this is unnecessary. Most favorable incubation temperature 39° to 40° C.

Amœba Agar (Musgrave and Clegg[3]).—Agar 1 per cent., tap-water 90 per cent., ordinary nutrient broth 10 per cent. Mix and sterilize as usual. Reaction about neutral to phenolphthalein.

Medium for Pure Cultures of Amœbæ (Williams).[4]—Fresh sterile brain, liver or kidney is cut in small pieces and placed on the surface of amœba agar.

Special Media for Yeasts and Moulds.—Most of the pathogenic yeasts and moulds can be cultivated on the media already described. Growth is much improved if glucose or maltose is added. The reaction should, however, be slightly acid or at most slightly alkaline to litmus.

For moulds, especially those infecting the skin, an agar containing no meat but 1 to 2 per cent. peptone and 2 per cent. of glucose or maltose and 0.5 per cent. of glycerin with no change of reaction (*Sabouraud*[5]) gives a very favorable medium.

For yeasts, beerwort media are especially favorable. Hopped beerwort is obtained at a brewery. Autoclave, cool and filter and then tube (this avoids subsequent precipitation). For solid media add 10 per cent. gelatin or 1.5 per cent. agar. Various carbohydrates may be added in 2 per cent. amounts.

CULTIVATION OF MICROÖRGANISMS.

Microörganisms can seldom be identified by the microscope alone. By this method certain characteristics, such as individual form, arrangement, individual reaction to stain, and motility or lack of motility can be studied. But in order to learn all the characteristics of an organism,

[1] Contribution to Med. Res., dedicated to V. C. Vaughan, Ann Arbor, 1903, p. 549. also Jour. Infect. Dis., 1904, **1**, 1.

[2] Jour. Exper. Med., 1912, **16**, 567.

[3] Manila Bureau of Public Printing, 1904.

[4] Jour. Med. Res., 1911, **20**, 263.

[5] Ann. de dermat. et de syph., 1892 and 1893.

including its specific relation to disease, we must be able to grow it in pure culture, that is, apart from all other organisms, and determine its cultural characteristics on various media, its immunity reactions and its action in the animal body. Koch's postulates (see p. 22) were based on the ability to obtain pure cultures. In cultivating microörganisms it is not only necessary to supply the appropriate foodstuff, but also to have the appropriate conditions as to temperature, moisture, access of oxygen, etc.

When we make cultures of any material, we are likely to find that instead of only one variety of microörganism, there are a number present. If we grow the material in *fluid media*, we find that as the different varieties develop they spread through the medium and become hopelessly mixed; furthermore, the more vigorous varieties outgrow the feebler ones, which are thus lost. If, however, the microörganisms in the material be scattered through or on a *solid medium*, they may grow in separate colonies which may be visible to the naked eye or can be seen with a low-power lens. From each of these colonies a pure culture may be obtained.

Methods of Isolating Pure Cultures.—*Plating Methods.*—Two plating methods are employed: pour plates or streak plates. Agar or gelatin media are used. The medium is first rendered fluid: gelatin, by placing the tubes in warm water (35° to 40° C.); agar, by immersing the tubes in boiling water until fluid and then cooling the tubes to between 40° and 42° C. Agar melts completely only when heated to the boiling-point of water or thereabouts and hardens as the temperature falls below 40° C. The cooling of the agar is necessary, otherwise the organisms would be injured by the heat. If enriching fluids are to be used, they are added to the agar when between 45° and 50° C. The material from which we wish to obtain pure cultures is then inoculated into the fluidified media. The method of inoculation (see Methods of Inoculating Culture Media, p. 144) is in general as follows: A loopful of the material is carried into a tube of the medium and thoroughly mixed with the medium by tilting and rolling the tube. Agitation sufficient to produce bubbles must be avoided. From this tube three loops are carried over to a second tube which is mixed in the same manner. From the second tube five loops are carried to a third tube and mixed. The lips of the tubes are flamed and the contents of each poured into a Petri dish, which is, if necessary, tilted to spread the agar evenly over the bottom of the dish. In pouring, the lid of the dish is raised just sufficiently to pour the contents of the tube into the dish, the lid acting as a protection against falling dust. In diluting the material with culture media, it is our object to separate the organisms so that when colonies develop from them they will be well separated, that is, *discrete*. If they are not well separated, we should touch several colonies in attempting to transfer the growth of one and thus fail to obtain pure cultures. As we have no way of telling what dilution will give us well-separated colonies, a series of dilutions is necessary. The method will have to vary with the probable number of organisms in the material. If they are in

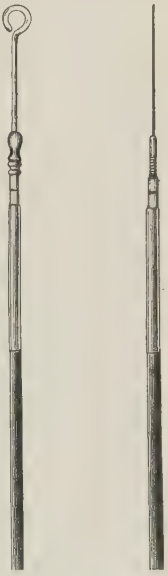


FIG. 48. — Platinum needle and loop. For most purposes finer wire is used.

great numbers, further dilutions of the material will be necessary, as the first tube will give overcrowded plates. A preliminary dilution or two may be made in saline solution or broth. When the material contains very few organisms, more material must be inoculated into the first tube. The dilutions may be made with a sterile pipette instead of a loop. The tip of the pipette is touched to the material and carried over to the first tube of medium, mixing being done by sucking the medium up and down in the pipette; about 0.1 c.c. of the contents of this tube is carried to a second tube, 0.1 c.c. of this to a third, etc.

Streak Plates.—In this method the medium (agar) is poured into Petri dishes and allowed to harden. The material is then streaked over the surface of the medium either with a platinum loop or spatula or a sterile bent-glass rod. Two or more plates are inoculated in succession without further sterilization of the loop or spatula. This dilutes the material so that discrete colonies are obtained. A difficulty that is likely to arise in the use of streak plates, and to a less degree with pour plates, is collection of water of condensation, which, finding its way to the surface of the plate, will cause a spreading of the growth over the whole surface. This can be avoided to a large extent by cooling the agar before pouring it in the plates. Special covers made of porous earthenware can be obtained, which will absorb the moisture. Incubating the plates upside down is a good precaution. Motile bacteria are most likely to spread over the surface of media. The special media used for the isolation of fecal bacteria must, because of the great numbers of motile bacteria in the stool, be comparatively dry. As most of these media restrain the ordinary contaminating bacteria from growing, they may be allowed to harden and dry in the Petri dishes with the covers off, and the drying continued by placing them in the incubator partly open.

Fishing Colonies.—This is the term used for the process of transferring the microörganisms from the colony to a fresh medium. This is usually done with a straight platinum wire, which is less likely to touch any colony other than the one we wish to fish. Fishing is done either by selecting the colony with the unaided eye or by the aid of a microscope. The advantage gained by the use of the microscope is that we can be sure that the colony to be fished has no microscopic colonies in the immediate neighborhood likely to be touched by the platinum wire. Furthermore, if the fishing is done under the microscope, the fact that one and only one colony has been touched is visually proved. The colony to be fished is centered under a No. 2 or AA objective and the wire introduced between the objective and the agar until the tip of the wire touches the colony. The progress of the wire is watched through the microscope to be sure that nothing is touched but the colony, and to observe by the broken appearance of the colony that it has been touched. The sense of touch is relied on to tell whether the objective is encountered. Opaque media are fished by eye. The plate is held against the light and the colony touched with the wire. In either case the organism is transferred to a fresh medium—usually an agar slant—by rubbing the tip of the wire over the surface of the medium.

Possible Sources of Error in Plating Methods.—Theoretically, each colony is the progeny of one single organism, or in the case of organisms that adhere to form pairs, chains, etc., the progeny of such an aggregate of one variety of organism. Practically it happens not infrequently that dissimilar varieties cling together and a colony develops containing both. A second plating from such a colony is usually sufficient to yield pure cultures. In exceptional instances there is the greatest difficulty in separating such "clingers" and it is sometimes impossible.

Special Methods of Isolation.—*Dilution Method.*—It is conceivable by gradual dilution of material to reach a point at which some of the loops of the material may only contain one organism which when carried to tubes of medium will yield pure cultures. This method was employed before the introduction of solid media. The uncertainty of the method is evident. For certain purposes modified dilutions are still employed, starting usually with cultures obtained

from colonies on plates, the method being employed not so much to obtain pure cultures as to obtain cultures known to be the progeny of one cell.

Hansen's Method for Yeasts.—Cover-slips are given a thin coating of paraffin, through which lines are drawn dividing the cover into small squares and each numbered. The marking is done with a needle. The covers are now exposed to hydrofluoric acid to etch the markings into the glass. They are then cleaned and sterilized. Dilute the yeast-containing material with fluidified gelatin until one loop averages one yeast cell as determined by microscopic examination. The gelatin is then spread over the surface of the sterile cover-slip, hardened and inverted over a Böttcher moist chamber, sealing with vaselin. This is placed under the microscope and the position of the single cells determined, making a sketch for later reference. Incubate at room temperature and when colonies have developed fish those developing from single cells. The advantage of this method over plating is that on plates a proportion of the colonies will develop from adhering cells. The purity of yeasts in industries is of enormous financial importance.

India-ink Method (Burri)[1]. —The use of india-ink for microscopic examination has already been described. By making successive dilutions of small amounts of culture in drops of ink it is possible to make small spots of ink containing only one organism. This is determined microscopically. This is the starting-point for the pure culture from one cell. The reader is referred to the article by Burri for the technical details. The method has only a very limited application (see page 77).

Capillary Method (Barber[2] and others).—Reference is made to the original articles for the details of this method for isolating one cell.

Preliminary Enrichment Methods.—Very often it is impossible to isolate certain organisms from material because they are too few in number. With certain organisms it is possible to inoculate the material in special media which will allow free multiplication of this organism, but be unfavorable for the others contained in the material. Examples of this are the use of Löffler's medium for mixed cultures from the throat to find diphtheria, or the peptone method for the enrichment of cholera and other vibrios. Another method is the use of ascitic broth for the isolation of diphtheria from mixed material. The last two methods take advantage of the surface growth of the bacteria. The same end is attained by the use of the following factors: Blood media for hemoglobinophilic bacilli; increase of the alkalinity, as in selective media for vibrios; increased acidity, as with tartaric acid, for yeasts; dyestuffs, gentian violet to inhibit Gram-positive bacteria, brilliant green to inhibit the colon types in mixed cultures from stools; use of heat, spore-containing mixed cultures being heated to 80° C., to kill the non-spore-bearing types; motility, the motile organism spreading from the point of inoculation of the mixed material, as with amœbæ and spirochete-containing material; filtration through filters, spirochetes passing filters, most bacteria being held back (see Filtrable Viruses); germicidal agents (see Anilin Dyes, p. 129); antiformin for separation of acid-fasts from other bacteria (see Tuberculosis, Chapter XXVIII); finally, the use of animals (same chapter).

Special Methods for Anaërobes.—(See Anaërobic Methods.)

Applications of the Plating Methods.—*Study of Colony.*—The appearance of the colony is one of the points used in identifying bacteria; or the colony appearance being known, the isolation of a certain organism is greatly simplified. In plates showing two or more types of colonies it is an aid in determining the relative proportion of the subsequently identified varieties in the material examined.

In examining colonies both the macroscopic and microscopic characteristics are determined. The age at which a colony will be most characteristic depends on the rapidity of growth of the organism. Daily observations are most suited

[1] Das Tuscheverfahren als einfaches Mittel, etc., Jena, G. Fisher, 1909.

[2] Kansas Univ. Sc. Bull., 1907, **4**, 41; also Jour. Infect. Dis., 1908, **5**, 380.

for full information. As a rule young colonies are most characteristic, although such characteristics as pigment production and gelatin liquefaction may only appear on longer incubation. With the naked eye the following points should be observed: Moist or dry, transparent, translucent or opaque, edge sharply defined or indefinite, regular or irregular outline, fringe-like margin or thread-like outgrowths, color, flat, raised or umbilicated, etc. On microscopic examination the presence or absence of granules, whether fine or coarse, their

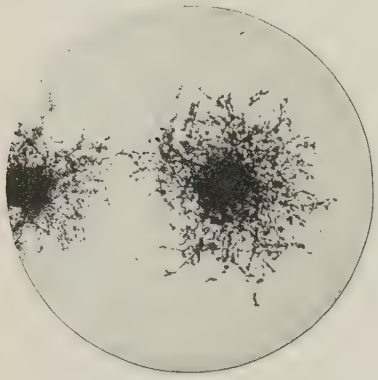


Fig. 49.—Irregular fringed colony (B. malignant edema). (From Kolle and Wassermann.)

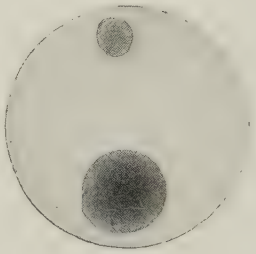


Fig. 50.—Colonies of typhoid and colon bacilli in rather soft gelatin.

arrangement and distribution, should be observed, as well as the finer details of the characteristics mentioned under the naked-eye examination. The observations are usually made with a low-power objective, No. 2 or No. 3. The fine details may be observed with a higher lens and the arrangement of the bacteria especially at the edge of the colony can be observed. The accompanying figures illustrate the colony characteristics mentioned.

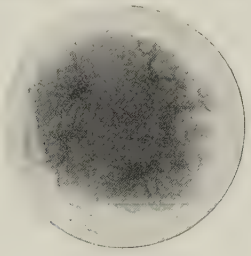


Fig. 51.—Colony of colon bacilli grown in soft gelatin.

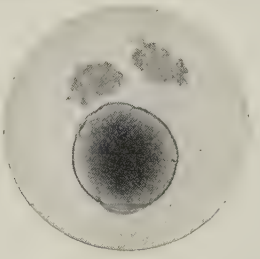


Fig. 52.—One large irregular colony of colon and two smaller colonies of typhoid bacilli in soft gelatin. (Figs. 50 to 52 from photographs by Dunham.)

Determination of the Number of Bacteria.—If a measured amount of fluid is mixed with a plating medium we should theoretically be able to determine the number of bacteria by counting the number of colonies that develop. This would be true if each bacterium was completely separate, if each was able to multiply, if the medium was satisfactory for the development of each variety contained in the material, and if the oxygen access, moisture and temperature of incubation were favorable for the growth of all the varieties. This is an impossibility. Most nearly accurate results are obtained where only one or at most several varieties are present and the conditions for their growth main-

tained. Although the method gives an incorrect result when mixed material is used, the enumeration of the colonies that develop is of a great deal of practical value, as in the examination of milk and water. If the methods employed are always the same, the comparison of the results gives us valuable information as to the bacterial content.

FIG. 53.—Moist raised colonies with no visible structure, looking like a drop of water. (Figs. 53–60 from Lehman and Neumann.)

FIG. 54.—Deep colonies, usually either light brown, gray or yellow in color, opaque with little marking.

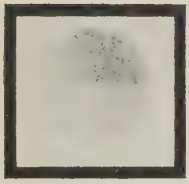


FIG. 55.—The colony very finely granular.

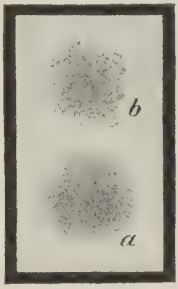


FIG. 56.—Colonies opaque in center with lighter borders. The margin is coarsely granular, or has twisted threads.

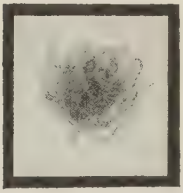


FIG. 57.—Colony in gelatin. The center is coarsely granular in partly fluid gelatin. The borders are formed of wavy bands of threads.

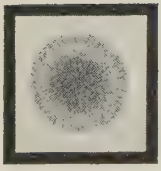


FIG. 58.—Colonies circular in form, composed of radiating threads.

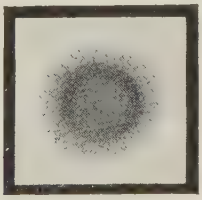


FIG. 59.—Colonies with opaque centers, with a thin border fringe.

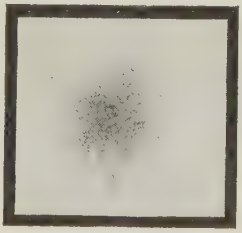


FIG. 60.—Colony showing a network of threads which is thicker in the center.

The method employed is in general as follows: To 9 c.c. of water is added 1 c.c. of the material to be examined; this is thoroughly shaken to separate the organisms; 1 c.c. of this is then added to 9 c.c. of water, thoroughly shaken, and 1 c.c. of this added to 9 c.c. of water and so on. This gives a dilution of 1 to 10, 1 to 100, 1 to 1000, etc. How far to dilute the material must be deter-

mined from the character and probable bacterial content. One c.c. of each of these dilutions is placed in a Petri dish and the melted plating medium poured into the dish, the two being mixed by tilting the dish. Mixing can be done by adding the dilutions to the tubes of plating medium and pouring the content into the dishes; but as all the medium cannot be poured out of the

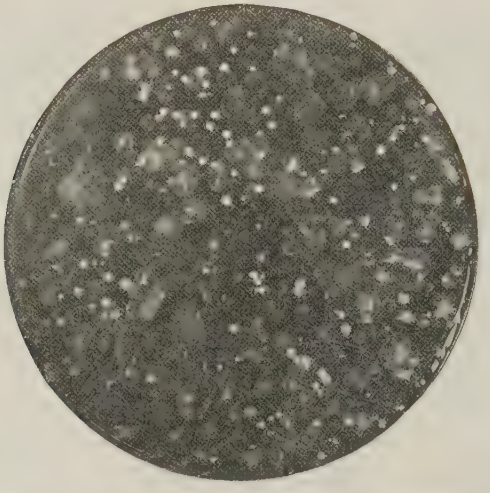


FIG. 61.—Photograph of a large number of colonies developing in a layer of gelatin contained in a small Petri dish. Some colonies are only pin-point in size; some as large as the end of a pencil. The colonies here appear in their actual size.

tube, some bacteria are left behind. It is easier, however, to obtain even distribution of the colonies in this way, and for comparative work the method gives reliable results. The counts by this method average about 10 per cent. lower than where mixing is done in the dish.

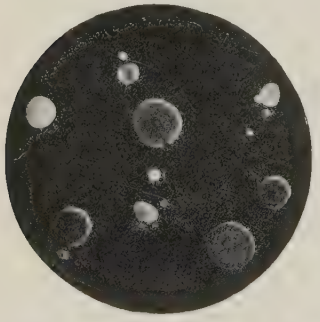


FIG. 62.—Well-distributed colonies in agar in portion of Petri dish.

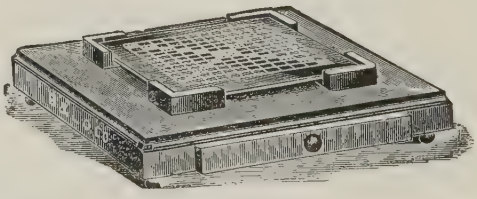


FIG. 63.—Wolffhügel's apparatus for counting colonies.

In place of dilutions made by pipettes, a series of loops or rings on a platinum needle can be employed which hold a known quantity (small) of the material to be examined.

To obtain a satisfactory count the colonies should be about 100 to 200 to a plate. Lower numbers than this are too few to give a fair average, higher numbers are difficult to count because the colonies have not sufficient space for full development, some of the colonies become confluent, and some of the

feebler bacteria are checked in their development by the crowding and do not develop colonies. The plate that shows about 100 colonies is chosen, therefore,

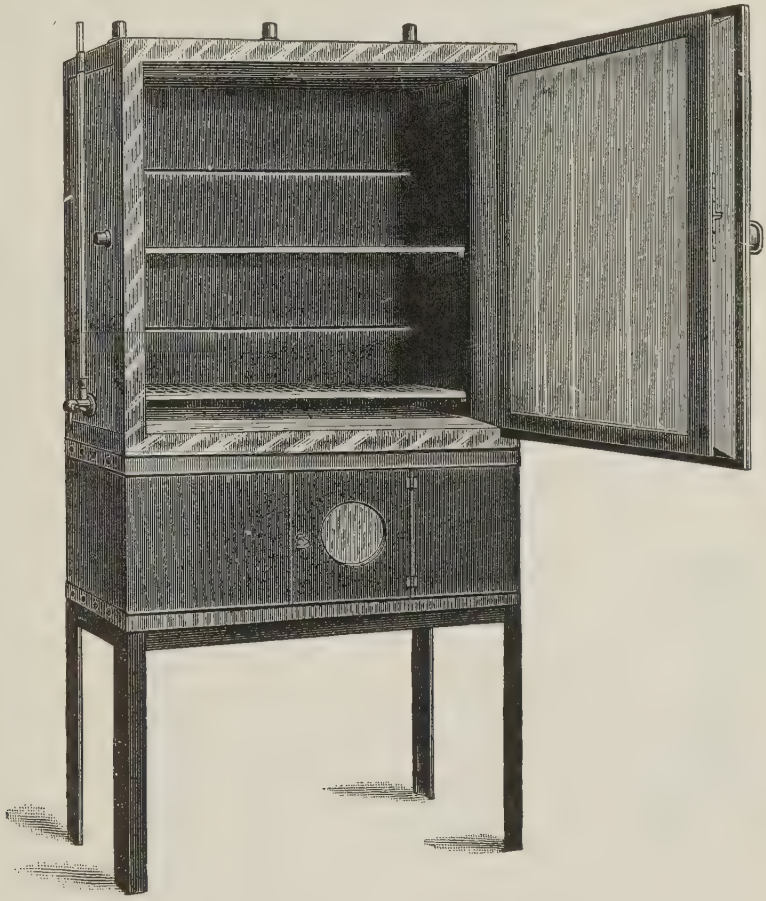


FIG. 64.—Incubator.

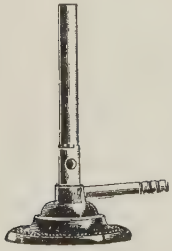


FIG. 65.—Bunsen burner, ordinary type.

for counting. When no one plate is completely satisfactory in number and even distribution of the colonies, two of the nearest satisfactory plates are counted and the results averaged. Whenever possible all the colonies on a

plate should be counted. Where the colonies are crowded it is necessary to divide the plate into sections to facilitate counting. This is best done by placing the Petri dish on a Wolffhügel counting plate, which is a glass plate ruled in square centimeters, some of the squares being still further divided into 9 small squares. A row of squares is counted and then a row at right angles to the first, to obtain a fair average. The number of colonies is divided by the number of squares counted. If the usual 9 c.m. Petri dish is used, the number per square centimeter is multipled by 63, the number of square centimeters on the dish, to give the total number. This is multiplied by the dilution used in this dish, which gives the colonies developing from 1 c.c. of the material used for plating. If dishes of another size are used, the area in square centimeters is determined by multiplying the square of the radius by 3.1416.

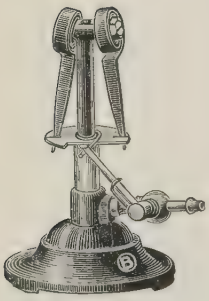


Fig. 66.—Safety-burner.

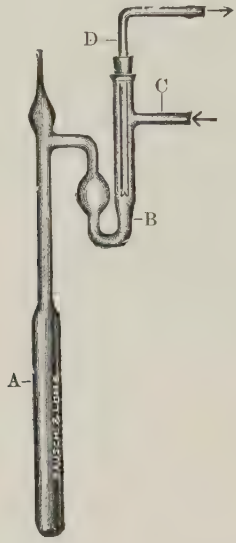


Fig. 67.—Heat regulator. (Dunham.)

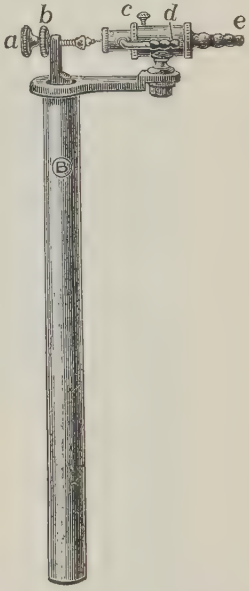


Fig. 68.—Heat regulator (Roux.) Bimetallic.

Methods of Inoculating Culture Media.—The inoculation of plating media has been described under the methods of isolating pure cultures. Platinum wire is usually employed for transferring the growth from one medium to another. The wire is either attached to the end of a glass rod or to special aluminum holders which are made for this purpose. Straight wire or wire with the end bent into a loop is used. The wire is sterilized by heating it in the flame of a Bunsen burner until red-hot. Care must be taken that the wire is cool before it is used. The straight wire is used for fishing colonies or where tubed solid media is to be inoculated by long puncture through the media (stab culture). The loop is used for ordinary transfers. In transferring from solid media the loop is drawn upward over the medium, filling the loop with the growth; this is rubbed over the surface of solid media or in the case of fluid media against the glass at upper level of the fluid. In transferring fluid cultures a loop of the fluid is used. When the growth on fluid media is in the form of a pellicle it may be necessary to inoculate this pellicle so that it floats or growth may not take place. In transplanting, the tubes or other containers must not be kept open any longer than necessary or contamination is likely to occur. In

transferring from and to tubed medium the tubes are held by placing them against the palm and fingers of the left hand, grasping the tubes by the butt between the thumb and the palm, low enough so that the contents of the tube are not covered by the thumb. The right hand holding the sterilized platinum wire is used to remove the cotton plugs, one being grasped by the small finger and the other between the fourth and fifth fingers. After drawing out the plugs the necks of the tubes are flamed and the growth then transferred and the plugs replaced; the platinum wire is then sterilized at once before laying it down to mark the tubes. The marking is done usually with a wax pencil made for writing on glass. The plugs should be protected from dust and just before use should be moistened with a weak antiseptic.

Apparatus for and Methods of Incubation.—The selection of the temperature for incubation depends on the organism to be grown and the medium employed. Although the optimum temperature for growth may vary between 5° and 50° C. or more, the great majority of organisms will grow at either 22° or 37° C. These are the temperatures usually employed. For general purposes the former is obtained by incubation at room temperature. Where a more exact and uniform temperature is necessary an incubator must be employed. This is a double-walled oven, the space between the walls being filled with water; heat is supplied by gas or electricity and cooling by the addition of cold water. The latter is only necessary when the outside temperature rises above 22° C. The heating or the addition of water is controlled by an automatic regulator. To maintain a temperature of 37° C. a similar incubator is employed, the heating, however, being continuous; an automatic regulator controls the temperature. The heat-controlling devices are of varied construction, the expansion of a fluid or the change in shape of a bar of two unequally expanding metals is used to make and break the electric contact or open and close the gas vent. In the former mercury is used alone or at the point of control. When a large incubating room is required, a room can be heated with a large gas stove, a funnel being placed over the stove and connected with this a radiator-like arrangement of 4- to 6-inch pipes ending in a chimney-like outlet for the products of combustion. The radiator distributes the heat more evenly through the room. In spite of this there will be a variation in temperature in different parts of the room, the shelves near the radiator or nearest the ceiling being the warmest. This is an advantage rather than a disadvantage, as a range of about 5° is available for special purposes. In using gas, all connections are best made of metal; flexible metal tubing is available. Small burners are preferably of the safety type, which, if extinguished, turn off the gas automatically. In the case of gas stoves there should be an independent pilot flame not influenced by the regulator, which will relight the stove in case of accident.

Oxygen Requirement Methods.—Where aërobic conditions are required the access of air to the growth is all that is necessary. For anaërobic conditions the oxygen must be removed. This is done (1) by exhaustion of the air, (2) by chemical absorption of the oxygen, (3) by displacement of the air as by hydrogen, or (4) by growing in the depths of solid media, or in fluid media under a layer of albolene to prevent the reabsorption of air driven off during sterilization; (5) or, lastly, by a combination of these methods. The addition of fresh animal tissues to media for the enhancement of the growth of anaërobic organisms has been mentioned under Media. Occasionally reducing substances are added directly to the medium, or the organism is grown in symbiosis with an aërobic bacillus which absorbs the oxygen.

The group of organisms spoken of as "micro-aërophilic" will only grow when the oxygen is partly removed. This may be done (1) by exhaustion of the air to measured degree, (2) by growing the organism in a sealed jar containing also a stated number of cultures of an organism that absorbs oxygen in its growth, as B. subtilis, or (3) by mixing the material with melted agar in tubes and allowing the agar to set, the development taking place at the depth that has the favorable oxygen content. In the second method the optimum number of square centimeters of B. subtilis culture per liter of air must be known.

Anaërobic Methods.—For exhaustion a pump is required; the filter pumps for attachment to the faucet are the most convenient where water under pressure is available. Any type of pump, however, may be employed. The extent of exhaustion can be measured by a manometer. Any type of jar of appropriate dimensions with a tight-fitting cover and a stop-cock opening can be used. The Novy jar is a satisfactory type. Museum jars with a side opening are very satisfactory substitutes. The jar must be absolutely tight or the vacuum will not be held. Absorption can be added to exhaustion by placing pyrogallic acid and a stick of sodium hydrate in the bottom of the jar. After exhaustion is completed a small amount of water is allowed to be sucked back into the jar. This dissolves the sodium hydrate and the resulting reaction of the sodium hydrate with the pyrogallic acid is accompanied by absorption of the oxygen from the air remaining in the jar.

Hydrogen may be used to replace the exhausted air. After exhaustion, hydrogen from a Kipp apparatus is sucked back into the jar diluting the remaining air; the jar is again exhausted and then filled with hydrogen, again exhausted and filled. Only traces of air will remain, and if absolute anaërobiosis is required pyrogallic acid may be used to absorb this last trace as above, allowing the water to be sucked back before the negative pressure is completely relieved by the hydrogen. In relieving the negative pressure it may be necessary to use a pneumatic trough to collect the hydrogen, as the generation of hydrogen will be too slow and air will be drawn through the Kipp apparatus. It is advisable not to relieve the negative pressure completely, as this helps to hold the lid tightly in place, and furthermore, the subsequent expansion when the jar is incubated will tend to loosen the lid and allow inward diffusion of air.

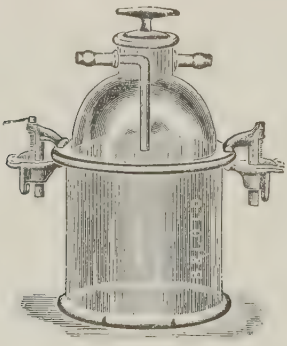


Fig. 69.—Novy jar for anaërobic cultures.

Fig. 70.—Buchner's anaërobic tube. The fluid consists of pyrogallic acid dissolved in 10 per cent. NaOH solution. By Wilson's method the tubes are charged with pieces of caustic potash covered with pyrogallic acid.

When the pyrogallic method is used, the tubes or plates must be raised above the level of the fluid. Of the methods given, the second and third are the most satisfactory. The absorption method may be used alone, using a jar to hold the tubes or plates; or individual plates or tubes may be handled as in the following:

Buchner's Method.[1]—The culture tube is placed in a larger tube, at the bottom of which is placed the pyrogallic acid, on which some sodium hydrate is poured and the tube quickly closed with a rubber stopper, or the NaOH may be put in dry and water added.

Wright's Method.[2]—The tubes are plugged with absorbent cotton; after inoculation of the medium the plug is pushed into the tube, pyrogallic placed on the stopper, sodium hydrate solution added, and the tube quickly corked.

Zinsser's Method for Plates.[3]—The dishes employed must be deeper than the ordinary Petri dishes. The agar is poured in the smaller dish in the ordinary manner, and as soon as hard inverted over moistened filter paper to protect it from contamination. In the larger dish is placed the pyrogallic acid and the smaller dish placed in it. One side of the smaller dish is raised and the hydrate solution poured in the space between the two dishes and the inner dish dropped back. As rapidly as possible albolene is run in the space between the dishes from a pipette previously filled so that no time is lost.

The methods of exclusion of oxygen are simple and can be, in most instances, substituted for the more cumbersome methods described. Plating may be done with the ordinary Petri dishes as follows (Krumwiede and Pratt[4]): The bottom part of the dish, open side up, is placed in the cover and sterilized in this shape and protected from dust until used. The plating medium is inoculated as though pour plates were to be made in the ordinary way. The inoculated medium is then poured into the cover and the bottom of the dish laid on the fluid medium, tilting it so that the air can escape as it falls into place. With a little practice this can be done so that no bubbles are present (see Fig. 71). After the medium has set the edge is painted over with melted paraffin to prevent drying and contamination. If any of the medium flows over the edge, it is wiped away by carbolized cotton. After incubation the parts of the dish are carefully pulled apart and the colonies may be fished. As a rule the agar remains in the cover. If it should adhere to the other part of the dish, this can be laid in the cover to allow of fishing under the microscope. If gas-producing bacteria are present, fishing should be done as soon as sufficient growth has taken place or the gas will disrupt the medium and cause the growth to spread over the plate. If an actively motile organism is present, the method may fail because of spreading between the medium and the glass.

FIG. 71.—Anaërobic plating method.

Another method for the isolation of pure cultures is the *shake culture.* The material is diluted with a plating medium in the usual way, although more medium is used in the tubes. Instead of pouring the medium into plates the medium is allowed to solidify in the tubes. The colony desired can be obtained as follows: scratch the tube with a file at the level of the colony, then wet the tube with alcohol, burn this off and press on the scratch with a very hot piece of metal or glass rod. The tube will crack and the column of agar can be cut

[1] Centralbl. f. Bakteriol., 1888, 1st Abt., vol. 4.
[2] Jour. Boston Soc. Med. Sci., 1900, 4, 114.
[3] Jour. Exper. Med., 1906, 8, 542.
[4] Jour. Infect. Dis., 1913, 12, 199.

through to allow the two parts of the tube to be separated and the colony fished. This method has a limited application, but is especially useful when the organism to be isolated is a partial anaërobe and grows at a certain level in the agar. Pure cultures of these organisms may not be obtained at the first attempt, but the level of thickest growth may then be used, the first culture serving in this way as an enrichment.

For the cultivation of pure cultures the use of stab cultures in agar or gelatin may be employed. The most satisfactory medium for this purpose is a semisolid agar (Krumwiede and Pratt). Because of the softness of the medium the material for subinoculation is easily obtained; the stab inoculation closes better than if made in stiffer medium, and the medium does not dry out nor split so readily. For subinoculation a platinum wire bent like a corkscrew is a help in obtaining the material from the depths of the media. With spirochetes it is much easier to obtain the growth with the capillary pipette from about the tissue at the bottom of the tube than where the usual 1.5 per cent. agar is employed. The use of the capillary pipette for the transfer of cultures is open to much wider application with semisolid media, because if the medium has a satisfactory density, the ordinary rubber teat exerts sufficient suction to draw up the medium and a syringe is not needed.

The *exclusion of oxygen* from a fluid medium is most simply carried out by sterilizing the medium under a layer of vaseline. This can be done either in tubes or flasks, preferably the globe flasks. In the latter the medium is filled to the neck and vaseline added, leaving room for expansion; after sterilization and contraction of the medium more sterile vaseline is added if necessary.

Occasionally anaërobes are grown in mixed culture with an aërobic organism to absorb the oxygen. One of the best organisms for this purpose is the hay bacillus.

As a general rule the addition of a fermentable carbohydrate to the medium gives much better growth of anaërobic organisms.

The Study of Pure Cultures.—The study of pure cultures is the basis for classification and identification. The characteristics studied are: the morphology, staining reactions and motility when grown on or in both solid and fluid media (see Microscopic Study); the cultural characteristics when grown on various media; the food, oxygen and temperature requirements; the reaction to the various immunity tests (see chapters on Immunity), and the action of the organism or its products on experimental animals (see Use of Animals). The following is a short summary of the more important cultural reactions and the methods of eliciting them.

Cultural Characteristics.—*Colony Morphology.*—(See Application of Plating Methods.)

Growth on Agar and Other Solid Media (*Surface Growth*).—Moist or dry, flat or raised, flaky or easily emulsified, adherent to medium or not, mucoid or slimy, smooth, irregular, or threaded margin, color or pigment production (see below), extension into medium, etc.

Growth in Solid Media.— In stab culture, depth to which growth extends, character of growth around the surface puncture, the spreading of the growth into the medium (an index of motility best shown in semisolid media), thread-like extensions from the line of puncture; in gelatin, liquefaction, non-liquefaction, rate, area and character if present; in shake cultures, the character of the colonies.

Fluid Media.—Pellicle, tenacious or easily broken, spreading up the side of the tube or not, thick or thin, clear or cloudy, and degree of clouding, sediment or deposit on sides of tube, granular, flaky, gelatinous, mucoid, or stringy, stalactite formation, more densely clouded at one level than another.

Food Requirements.—Growth on simple or complex media, albumins such as serum required, blood or hemoglobin necessary, carbohydrate needed for full development (example—glycerin for tubercle bacillus).

Oxygen Requirements.—Growth only in free oxygen, obligate aërobe; growth only where oxygen is absent (depths of media, etc.), obligate anaërobe; growth under both conditions, facultative anaërobe (an available carbohydrate is necessary to elicit the facultative character); growth with a definite but lessened amount of oxygen (development only in one level of solid media), micro-aërophilic. This last characteristic may be lost after cultivation for some time on artificial culture media.

FIG. 72.—Stab cultures of three cholera spirilla in gelatin, showing in upper portion of growth considerable liquefaction of nutrient gelatin.

Optimum Temperature.—Not often used, but may furnish absolute evidence of the dissimilarity of two organisms. Not only the optimum but also the range of temperature, especially lower, should be determined.

Pigment Production.—The color and shade, the optimum medium and temperature for its production, limited to the area of growth, or diffused through the medium; its solubility in extracting agents, as alcohol, ether, chloroform, etc.; more than one pigment present, as shown by use of different extractives. In the case of the coccaceæ especially, the presence of pigment and its color and shade may be determined with ordinary agar; the growth is taken up with a platinum loop and spread on a piece of white glazed paper. Although the presence of pigment may be completely obscured on the agar, its presence is immediately evident on the paper.

Ferment Action (Cultural Evidence of).—The most important culturally are those causing fermentation of carbohydrates. Less important are proteolytic, diastatic, inverting and rennin-like ferments.

Fermentation of Carbohydrates.—The sugars selected will depend on the organism to be identified as well as the medium selected as a basis for the addition of the sugar. The preparation and the use of indicators or titration to determine the production of acid and the use of fermentation tubes to demonstrate gas production have been described. The amount of gas is expressed in terms of percentage, thus if the closed arm is half-full of gas, 50 per cent., etc. To determine roughly the proportion of carbon dioxide and of hydrogen, mark on tube quantity of gas produced, then fill the bulb with sodium hydroxide solution (10 per cent.) and insert a rubber stopper; the tube is inverted several times to mix and the gas again collected in the closed arm. The gas absorbed is carbon dioxide; the remainder is usually hydrogen. In the case of media containing coagulable proteins (as in milk, serum water, serum broth), acid production, if sufficient, is followed by coagulation; the coagulation is shown in

solid media by precipitation or opacity. The ability to attack a carbohydrate may differ under aërobic and anaërobic conditions.

Proteolytic action is shown by gelatin liquefaction, liquefaction of Löffler's blood serum, digestion of milk. Prolonged incubation even for a month may be needed. The production of peptone may also be used for determining the digestion of albuminous media. Note odor and, if present, its character.

Alkali Production.—Determined by indicators or titration; note in milk cultures especially. An initial acidity may be noted, due to traces of a fermentable carbohydrate. The cultures may have to be incubated for one to three weeks. Milk on prolonged incubation may become translucent whether due to alkali production or to a proteolytic enzyme not determined.

Sulphuretted Hydrogen.—To demonstrate, use peptone water cultures to which is added 1 per cent. of a 1 per cent. solution of lead acetate or ferric tartrate; the precipitate which forms on mixing these turns black if sulphuretted hydrogen is produced.

Nitrite Production.—Grow in the nitrate broth (p. 122). Two solutions are necessary for the test.

1. Naphthylamin	0.1 gm.
Distilled water	20.0 c.c.
Acetic acid, 25 per cent.	150.0 c.c.

Dissolve the naphthylamin in the water by means of heat, cool, filter, and add the acetic acid.

2. Sulphanalic acid	0.5 gm.
Acetic acid dil. 1 to 16	150.0 c.c.

Keep them separate and mix in equal parts as needed.

To about 4 c.c. of the culture add 2 c.c. of the mixed solutions. The development of a pink color shows the presence of nitrites, the intensity of the color being proportional to amount present.

Similar reduction processes are seen in the decolorization of litmus and some of the aniline dyes. The bacteria utilize the oxygen of the dyes, reducing them to their leuco-bases.

Aromatic Products (*Indol Production*).—The usual medium is peptone water. Two methods are available for testing: (1) Salkowski Method. To the culture add several drops of concentrated sulphuric acid or 1 c.c. of a 10 per cent. solution and then add 1 c.c. of a 1 to 10.000 sodium nitrite solution. A pink color develops at the point of contact with the acid, which on shaking diffuses throughout. (2) Ehrlich Method. This test is more constant and reliable. To the culture add 1 c.c. of a 2 per cent. solution of paradimethylaminobenzaldehyde in 95 per cent. alcohol and then add, drop by drop, concentrated hydrochloric acid until a red zone appears at the point of contact of the alcohol and the peptone. Not more than 0.5 c.c. of the acid is required. On standing the zone deepens and widens. The color is soluble and the test should be confirmed by shaking with chloroform, which dissolves out the color. The tests for indol are made after four to six days' incubation. Kligler[1] gives a good résumé of the subject.

Cholera-red Reaction.—Nitrite is produced by some bacteria from the nitrate present as an impurity in the peptone, and the red color appears on the addition of acid alone. This test, therefore, is for indol production and nitrate reduction.

Both of the above tests are interfered with by the presence of a fermentable carbohydrate.

Vosges-Proskauer Reaction.[2]—Cultures are grown for two or three days at 37° C. in either dextrose broth or in dextrose potassium phosphate broth in fermen-

[1] Jour. Infect. Dis., 1914, **14**, 81.
[2] Ztschr. f. Hyg., 1898, **28**, 20.

Endorsed by SOCIETY OF AMERICAN BACTERIOLOGISTS at the annual meeting Dec 29, 1920

DESCRIPTIVE CHART

Prepared by H. J. CONN, K. N. ATKINS, I. J. KLIGLER, T. P. NORTON, C. E. HARMON. } Committee on bacteriological technic

Name of organism.....Source.....Date of isolation.....Studied by.....Culture No.....

INVIGORATION OF CULTURE: Date.....Medium used.....Temperature.....Number of transfers.....Length of each incubation....days. Series No....

MORPHOLOGY

NOTE—Underscore required terms. SKETCHES

VEGETATIVE CELLS, Medium used.....
temp..... age..... days.
Form, *spheres, short rods, long rods, filaments, commas, short spirals, long spirals, curved.*
Arrangement, *single, pairs, chains, fours, clusters, cubical packets.*
Limits of Size.....Size of Majority.....
Ends, *rounded, truncate, concave.*

CAPSULES, present on.....
How stained.....

SPORANGIA, *present, absent.* Medium used.....
temp..... age..... days.
Form, *elliptical, short rods, spindled, clavate, drumsticks.*
Limits of Size.....Size of Majority.....

ENDOSPORES, *present, absent.*
Location of Endospores, *central, polar.*
Form, *spherical, elliptical, elongated.*
Limits of Size.....
Size of Majority.....
Wall, *thick, thin.*
Sporangium wall, *adherent, not adherent*

MOTILITY
In broth..... On agar.....

FLAGELLA, No.....Attachment, *polar, bipolar, peritrichiate.* How stained.....

IRREGULAR FORMS.
Present on.....in....days at.....°C.
Form *spindled, coneate, filamentous, branched, or.....*

STAINING REACTIONS.
Gram.....Acid fast.....
Special stains.....
.....
.....
.....

CULTURAL CHARACTERISTICS

	Underscore required terms.	SKETCHES		Underscore required terms.
Agar Stroke Incubation Temperature .....°C Age .....d	Growth, *scanty, moderate, abundant, none.* Form of growth, *filiform, echinulate, beaded, spreading, arborescent, rhizoid.* Elevation of growth, *flat, effuse, raised, convex.* Lustre, *glistening, dull.* Texture, *smooth, contoured, rugose.* Optical Characters, *opaque, translucent, opalescent iridescent.* Chromogenesis.....Photogenic.....Fluorescent Odor, *absent, decided, resembling.....* Consistency, *butyrous, viscid, membranous, brittle.* Medium, *grayed, browned, reddened, blued, greened.*		Nutrient Broth Temperature .....°C Age .....d	Surface growth, *ring, pellicle, flocculent, membranous, none.* Clouding, *slight, moderate, strong, transient, persistent, none, fluid turbid* Odor, *absent, decided, resembling.....* Sediment, *compact, flocculent, granular flaky, viscid on agitation, abundant scant, none.*
Gelatin Stab Temperature .....°C Age .....d	Growth, *uniform, best at top, best at bottom.* Line of puncture, *filiform, beaded, papillate, villous arborescent.* Liquefaction, *none, crateriform, napiform, infundibuliform, saccate, stratiform, beginning.....complete in.....d* Depth of liquefaction in tube of 10 mm. diameter evenly inoculated at 20° C. for 20 days.....mm. Medium, *fluorescent, browned.*		Medium (liquid) Temperature .....°C Age .....d	
Medium (solid) Temperature .....°C Age .....d			Medium Temperature .....°C Age .....d	

Index No.*.....

BRIEF CHARACTERIZATION

As each of the following characteristics is determined, indicate in proper marginal square by means of figure, as designated below:

PRIMARY CHARACTERISTICS

Microscopic Features

Form: 1, streptococci; 2, diplococci; 3, micrococci; 4, sarcinæ; 5, rods; 6, commas; 7, spirals; 8, branched rods; 9, filamentous

Spores: 1, central; 2, polar; 3, absent

Flagella: 1, peritrichic; 2, polar; 3, absent

Gram stain: 1, [illegible]

Miscellaneous Biochemical Reactions

Pathogenicity, etc.: 1, [illegible]; 2, [illegible]; 3, [illegible]; 4, parasitic but not pathogenic; 5, saprophytic; 6, autotrophic

Relation to free oxygen: 1, [illegible]; 2, [illegible]; 3, [illegible]; 4, strict anaerobe

Gelatin liquefaction: 1, positive; 2, negative

Reduction of nitrate to nitrite: 1, nitrite and gas; 2, nitrite but no gas; 3, neither nitrite nor gas

Chromogenesis: 1, flourescent; 2, violet; 3, blue; 4, green; 5, yellow; 6, orange; 7, red; 8, brown; 9, [illegible]

Carbohydrate Reactions

Diastatic action: 1, positive; 2, negative

From dextrose: 1, acid and gas; 2, acid without gas; 3, no acid

From lactose: 1, acid and gas; 2, acid without gas; 3, no acid

From sucrose: 1, acid and gas; 2, acid without gas; 3, no acid

SECONDARY CHARACTERISTICS

Vegetative Cells

Diameter: 1, under 0.5μ; 2, between 0.5μ and 1μ; 3, over 1μ

Length: 1, less than 2 diameters; 2, more than 2 diameters

Chains (4 or more cells): 1, present; 2, absent

Capsules: 1, present; 2, absent

Spores

Shape: 1, round; 2, oval to cylindrical

Diameter: 1, less than diameter of rod; 2, greater than diameter of rod

Cultural Features

Agar Stroke

Abundance: 1, abundant; 2, moderate; 3, slight; 4, absent

Lustre: 1, glistening; 2, dull

Surface: 1, smooth; 2, contoured; 3, rugose

Agar colonies: 1, punctiform; 2, round (over 1 mm. diameter); 3, rhizoid; 4, filamentous; 5, curled

Gelatin colonies: 1, punctiform; 2, round (over 1 mm.); 3, irregular; 4, filamentous

Milk

Acid: 1, sufficient for curdling; 2, insufficient for curdling; 3, no acid

Rennet curd: 1, present; 2, absent

Peptonization: 1, present; 2, absent

*Recording the "Index Number" here is optional; but its use will be found convenient if the charts are to be filed according to the salient characteristics of the organisms. The Index Number consists of the first thirteen figures from the margin (primary characteristics) copied down in the order of their occurrence in the margin, placing a dash wherever a heavy rule occurs in the margin. Thus, B. coli belongs to the group 5312-41220-1111.

Agar Colonies Temperature .....°C Age .....d	Growth, *slow, rapid.* Form, *punctiform, circular, irregular, mycelioid, filamentous, rhizoid.* Surface, *smooth, rough, concentrically ringed, radiate.* Elevation, *flat, effuse, raised, convex, pulvinate, umbonate.* Edge, *entire, undulate, lobate, erose, filamentous, curled.* Internal structure, *amorphous, finely-, coarsely-granular, filamentous, curled, concentric.*	SKETCHES	Gelatin Colonies Temperature .....°C Age .....d	Growth, *slow, rapid.* Form, *punctiform, circular, irregular, mycelioid, filamentous.* Elevation, *flat, raised, convex, pulvinate, crateriform (liquefying).* Edge, *entire, undulate, lobate, erose, filamentous, floccose, curled.* Liquefaction, *cup, saucer, spreading.* Internal structure, *amorphous, finely-, coarsely-granular, filamentous, curled, concentric.*	SKETCHES

PHYSIOLOGY

TEMPERATURE RELATIONS

Optimum temperature for growth.....°C.
Maximum temperature for growth.....°C.
Minimum temperature for growth.....°C.

CHROMOGENESIS

Nutrient broth.....
Nutrient gelatin.....
Nutrient agar.....
Potato.....

PRODUCTION OF INDOL

Medium:.....
Indol *absent, present in*.....*days*

PRODUCTION OF HYDROGEN SULFIDE

Medium:.....
H.S *absent, present in*.....*days*

RELATION TO OXYGEN

Method used.....
Medium .....Temperature .....°C
Aerobic growth; *absent present, better than anaerobic growth.*
Anaerobic growth: *absent, occurs in presence of dextrose, of sucrose, of lactose, of nitrate; better than aerobic growth.*

DIASTATIC ACTION

Breadth of clear zone on starch agar plates.
in.....days:.....
Additional data.....
.....
.....

MILK

Temperature .....°C.

Reaction:	1 day.....	2 days.....	4 days.....	7 days.....	10 days.....
Acid curd:	1 day.....	2 days.....	4 days.....	7 days.....	10 days.....
Rennet curd:	1 day.....	2 days.....	4 days.....	7 days.....	10 days.....
Peptonization:	1 day.....	2 days.....	4 days.....	7 days.....	10 days.....

Reduction of litmus in.....days; of methylene blue in.....days.
Influence of indicator on growth.....

NITRATE REDUCTION

Medium..... Temperature.....°C.

Nitrite:	1 day.....	2 days.....	4 days.....	7 days.....	10 days.....
Gas:	1 day.....	2 days.....	4 days.....	7 days.....	10 days.....

Medium ..... Temperature.....°C.

Nitrite:	1 day.....	2 days.....	4 days.....	7 days.....	10 days.....
Gas:	1 day.....	2 days.....	4 days.....	7 days.....	10 days.....

FERMENTATION

Temperature.....°C.

Medium containing .... and:	DEXTROSE	LACTOSE	SACCHAROSE	GLYCERIN		
Gas						
First appearance of acid						
First appearance of alkali.....						
Reaction after.....days						
Reaction after.....days						
Max. H-ion Conc.....						

SPECIAL TESTS (e. g. PATHOGENICITY)

tation tubes. Add 1 c.c. of a strong 50 per cent. solution of potassium hydroxide solution and leave at room temperature for twenty-four hours. A positive reaction appears as an eosin pink color at the top of the tube, which is exposed to the air, due to the presence of acetyl-methyl-carbinol.[1] Boiling 1 to 4 c.c. of the culture with 5 c.c. of a 10 per cent. caustic soda solution is a quick method of obtaining the reaction. By the sugar alone, a yellow color is produced under these conditions when the test is negative. Shaking or blowing into the tube to promote oxidation hastens the reaction.

Rettger[2] cultivates the organisms for twenty-four hours and then tests by using equal parts of 5 c.c. each, of culture and of 10 per cent. potassium hydroxide and incubates at 37° C. Frequent observations and shaking of the tubes during the day are necessary lest the detection of weak carbinol producers be missed. He claims that decidedly positive reactions can always be obtained in one to eight hours.

Toxin Production.—The presence of a toxin is not usually elicited by cultural methods; one exception is the demonstration of hemolysins by plating with blood agar. The hemolysis is shown by a clearing about the colony.

For other methods of demonstrating the production of toxin, and the use of immunological reactions and animal tests see the appropriate sections of this work.

Chart.—The accompanying chart is given as an example of a relatively complete cultural study of an organism and the methods of using cultural reaction for classification. The characteristics to be elicited will depend on the group of organism studied. In the case of many of the organisms pathogenic for man and animal, cultural characteristics give us only a presumptive identification, the final identification depending on animal and immunological tests.

[1] Prescott and Winslow, 1913, 3d ed., p. 181.
[2] Personal communication from Dr. Rettger.

CHAPTER V.

THE USE OF ANIMALS FOR TEST PURPOSES.

Suitable animals are necessarily employed for the following purposes:[1]

I. To obtain growth.
   1. When organism grows with difficulty on artificial media or
   2. When organism takes hold of artificial media poorly.
   3. When we wish to obtain an organism in pure culture from contaminated material or mixed infections, *e. g.*
      (*a*) Material suspected of containing tubercle bacilli is injected into guinea-pigs where tubercle bacilli usually grow abundantly and without other organisms, particularly in lymph nodes and spleen.
      (*b*) Pneumococci in sputum or other material containing a mixture of microörganisms, when put into the peritoneum of a mouse, grow more quickly than do the other microörganisms and we are able thus in a short time to get purer pneumococci for cultures and other tests.

II. To maintain microbes that have not been grown on artificial media.
   1. A few bacteria.
   2. Some protozoa.
   3. Filtrable viruses, etc., *e. g.* Rabies, vaccinia, poliomyelitis viruses.

III. To test virulence or toxicity.
   1. Invasive virulence, *e. g.*, B. pestis, etc.
   2. Toxicity, *e. g.*, B. diphtheria, B. tetani.

IV. To produce serums containing antibodies, to be used for diagnostic or therapeutic purposes.

V. To raise virulence of an organism, *e. g.*, streptococcus, or a virus, *e. g.*, rabies.

VI. To test the activity (protective power) of antibacterial serums (pneumococcus serum) or of antitoxic serums (diphtheria antitoxin).

To obtain a growth of varieties that for any reason grow with difficulty on artificial culture media, as in the case of tubercle bacilli, material suspected of containing the bacilli is injected into guinea-pigs, with the knowledge that, if present, although in too small numbers to be detected by microscopic or culture methods, the bacilli will develop

[1] Selected examples are given. Further examples will be noted in the chapters on Specific Bacteria, Protozoa and Viruses.

in the susceptible animals' bodies, and thus reveal themselves, whereas any associated contaminating bacteria will be destroyed, unless they are virulent as well. If this is the case the method may fail and the material will have to be treated to kill these associated virulent organisms. (See Tuberculosis.) Similar difficulties may be met in other methods, for instance, a virulent influenza bacillus in sputum may interfere in pneumococcus work. Certain microörganisms have not yet been grown on artificial media. This is true of a few bacteria, of most protozoa, of many of the spirochetes and of certain unknown infectious agents such as produce smallpox and Rocky Mountain spotted fever. Animals are used to test the virulence or toxin production of organisms. In the case of diphtheria or tetanus the injection into a susceptible animal, such as the guinea-pig, is the only way that we can differentiate between those capable of producing diseases and those which are harmless. To test the antitoxic or microbicidal strength of sera, an antitoxic or antimicrobal serum is added to its respective toxin or microbe and injected into an appropriate animal.

The Inoculation of Animals.—The inoculation of animals may be made by any one or more of the following methods:

1. Cutaneous. The material is merely placed upon or rubbed into the abraded skin.

2. Intracutaneous. The material is injected into the skin. Important specific local reactions may be obtained by this method.

3. Subcutaneous. The substances are injected by means of a hypodermic syringe under the skin, or are introduced by a platinum loop into a pocket made by an incision.

4. Intravenous. The substances are injected by means of a hypodermic needle into the vein. In the rabbit the ear vein is chosen. If rabbits are placed in a holder, so that the animal remains quiet and only the head projects, it is usually easy to pass a small needle directly into one of the ear veins, especially those running along the edge of the ear. If the ear is first moistened with a 3 per cent. carbolic acid solution, and then supported between the finger inside and the thumb outside, the vein is usually clearly seen and entered with ease, if a small, sharp needle is held almost parallel with the ear surface and gently pushed into it. When no holder is obtainable the rabbit can be held by an assistant's seizing the forelegs in one hand and the hind in another and holding the rabbit head downward, or the animal may be held between the knees of the operator, its body resting on the operator's apron.

5. Into the heart. The smaller animals (guinea-pigs) may be inoculated directly into the heart by means of a hypodermic syringe.

6. Into the anterior chamber of the eye. Corneal inoculation is also employed.

7. Into the body cavities. The peritoneal and less often the pleural cavities are used for microbal injection. The hypodermic needle is usually employed, less often a glass tube drawn out to a fine point. The needle or the pointed glass tube is gently pushed through the

abdominal wall, moved about to be certain that the intestines have not been perforated and the fluid injected.

8. By inhalation. This method is carried out by forcing the animal to inhale an infected spray or dust.

9. By the trachea. This method is carried out either by making an incision in the trachea and then inoculating the mucous membrane or injecting substances into the trachea and bronchi, or by passing a catheter through the mouth and larynx into the trachea.

10. Through the intestinal tract by swallowing or by the passage of a rubber tube. Morphine may be given to prevent peristalsis.

11. Into the brain substance or ventricles after trephining, or when the parietal bones are thin, as in the guinea-pig and the rabbit, after making a tiny opening with the point of a small, heavy scalpel.

12. Intraneurally.

13. Intraspinously.

Mice, which are frequently inoculated at the root of the tail, are best placed in a mouse holder, but can be inoculated by grasping the tail in a pair of forceps, and then, while allowing the mouse to hang head downward in a jar, a glass plate is pushed across the top until only space for its tail is left.

Monkeys and apes are used for the inoculation of certain infectious material, such as from syphilis, poliomyelitis and smallpox, where only they and man are characteristically susceptible.

All these methods must be carried out with the greatest care as to cleanliness, the hair being clipped and the skin disinfected with tincture of iodine, then alcohol. The operator must be careful not to infect himself or his surroundings. After the inoculations the animals should be given the required food and kept in appropriate quarters. For food, rabbits and guinea-pigs are usually given carrots and hay. Mice may be fed puppy biscuits or a mixture of grains. Under special circumstances special diets may be given. All animals should be anesthetized during painful experiments.

Autopsies.—Autopsies should be made at the earliest moment possible, for soon after death some of the species of the bacteria in the intestines are able to penetrate through the intestinal walls and infect the body tissues. If delay is unavoidable, the animals should be put immediately in a place where the temperature is near the freezing-point. In making cultures from the dead bodies the greatest care should be taken to avoid contamination. The skin should be disinfected, and any dust or loose hair prevented by wetting with a 5 per cent. solution of carbolic acid. All instruments are sterilized by boiling in 3 per cent. washing-soda solution for twenty minutes. Changes of knives, scissors, and forceps should be made after cutting and skinning, after opening each cavity and after handling each organ or tissue. When organs are examined, and there is danger that the surrounding cavity is infected, the portion of the surface through which an incision is to be made must be sterilized by searing with the flat blade of an iron spatula which has been heated to a dull red heat. Tissues if removed should be immediately placed

under cover so as not to become contaminated. Sterile deep Petri plates are useful for this purpose.

When it is necessary to transport tissues from a distance, they should be put in tight sterile containers, *well labeled*, and sent to the point of destination as soon as possible. In warm weather they may be kept cool by surrounding the vessels which contain them with ice.

Animals may not show the same gross lesions as man when both suffer from the same infection.[1] The cell changes, however, are similar, and, also, so far as we can test them, the curative or immunizing effects of protective sèrums.

Animals comparatively frequently undergo spontaneous infections with organisms similar to those producing disease in man.[2] This is particularly so with the streptococcus-pneumococcus group and the paratyphoid group, so one must be constantly on one's guard in working with such organisms.[3] In obtaining cultures by use of animals or in raising virulence, one must be constantly on one's guard against possible substitution through the natural infections of the animals. In carrying out tests for toxin or toxin-antitoxin neutralization, etc., one must not forget that death may be due to or hastened by spontaneous infection.

The greatest possible care must be taken in identification of the animals used, most especially where the tests are made to determine the strength and purity of biological products. Separate jars or cages, staining the skin at different places, clipping the hair, tags in the ear or both ears, descriptions of color and markings, tattooing, tags on collars, etc., may be employed. The longer the animals are to be held the more permanent must be the means of identification. The mere separation by location, jar or cage, is the weakest method.

[1] Smith, Theobald: Factors in Pneumonia of Lower Animals, Jour. Med. Res., 1913, **29**, 291.

[2] Meyer, K. F.: Communicable Diseases of Laboratory Animals in Newer Knowledge, etc. Edited by Jordan and Falk, Univ. of Chicago Press, 1928, p. 607.

[3] Holman, W. J.: Natural or Spontaneous Infection in Animals, Jour. Med. Res., 1916, **35**, 152. (With bibliography.)

CHAPTER VI.

THE PROCURING AND HANDLING OF MATERIAL FOR MICROBIOLOGICAL EXAMINATION FROM THOSE SUFFERING FROM DISEASE.

A LONG experience has taught us that physicians very frequently take a great amount of trouble and yet, on account of not carrying out certain simple but necessary precautions, make worthless cultures or send material almost useless for microbiological study.

In making laboratory examinations of diseased tissues various procedures may be carried out, according to the nature of the infection and the kind of information that may be desired.

Obtaining Material from the Living Subject.—In the case of local areas, such as abscesses, the most desirable time to obtain material is before spontaneous opening with discharge occurs. In the case of superficial abscesses the skin should be sterilized and then incised. In the case of deeper areas or organs the surgeon will obtain the material at operation or may excise the infected area. The material may be collected by either of two methods, by sterile cotton swab or by sterile pipette. For the latter a sterile capillary pipette with rubber teat[1] is very convenient. If the swab tube or pipette outfit be sterilized in an outer tube it is convenient for the operating room, as the inner outfit may be slid out on the sterile instrument table for use by the surgeon.

Difficulties may arise where passage over or through areas which subject to contamination the instrument employed, *e. g.*, reaching the middle ear, the sinuses, the uterus, etc. Preliminary irrigation, the use of sterile specula, etc., may be employed as safeguards.

In the case of infection of the pleural, pericardial or abdominal cavities or of the meninges, material may be obtained by puncture, using a syringe and needle.

When spontaneous rupture or opening has occurred secondary infection or contamination is likely. It is well to clear away the collected discharge by irrigation with sterile saline and then collect the material that exudes. Curretting after cleansing and removal of superficial dead tissue may be desirable to obtain the infecting agent, as, for instance, in a tuberculous cavity or sinus.

Urine is preferably collected by sterile catheter. The urine direct from each ureter may be collected separately.

Sputum may be collected after coughing, remembering, however, that sputum passes over a contaminating route. Lung puncture has been employed. Feces may be collected in a clean (not necessarily sterile)

[1] This must be sterilized in the autoclave because of the rubber.

container. Blood for culture is discussed separately. (See below.) Blood smĕars (blood obtained by finger puncture) are especially used in the protozoan infections.

All containers (with the possible exception of those for sputum and feces) should be sterile and free of antiseptic.

If the materials are to be sent directly to a laboratory care should be taken against leakage and drying. In the case of feces or other material containing mixtures of organisms delay in examination may give negative or misleading results through multiplication of the more hardy types with the overgrowth and death of the specific pathogen sought. The examiner should receive with the materials, a statement of the conditions under which materials were obtained. These facts should be noted even if the examiner has collected the materials, or otherwise at some future date they may be forgotten and misleading information sent out. As full a history of the case as possible should be sent with the material. Especially the stage of the disease at which the material to be examined was gathered should be stated. This aids the examiner in deciding which method to use in his examination, and how to judge his results.

Handling and Examination of Materials.—The direct spread—in many instances a direct spread appropriately stained will give all the information needed from a practical standpoint. Thus in a spread of pus the presence of grape-like clusters of cocci means staphylococci, chained cocci, streptococci; cultural examination will verify this conclusion and if desired, add information as to the kind. In the case of discharges from gonorrheal infectious practical reliance is placed on the direct smear. Other examples are noted in the special chapters. In protozoan infections spread examinations are almost exclusively used. Special procedures requiring moist preparations will be noted under syphilis, amœbic dysentery, etc. The direct smear should always be used as a preliminary to cultures. It gives us a tentative idea of the numbers and kinds of microbes present and their relative proportion if more than one be present. It indicates the media to be used, the mode of inoculum, the degree of dilution required to obtain separate colonies, etc. It may give us information not otherwise obtainable; thus if aerobic cultures only are made from the pus in pyorrheal pockets streptococci will be the apparently predominating variety of microörganism, whereas direct smears will reveal the presence of many more fusiform bacilli and spirochetes than cocci, but these organisms are only obtainable by highly specialized methods of anaërobic cultivation. If the material from an otitis media be cultured direct only staphylococci may be recovered, whereas small numbers of streptococci may be visible in the smear, which could be obtained by enrichment methods. The smear and the source of the material will indicate whether animal inoculation should be resorted to.

There are only four conditions where the direct spread may be omitted: where the amount of material is so small as to render both smear and cultivation impossible; where experience has shown us that so few microorganisms are present as to be only demonstrable by cultures, thus in

the circulating blood, bacteria would only be demonstrable in film in exceptional instances, again, in certain forms of chronic infections of long standing, such as perialveolar abscess, very few organisms may be present; the third condition is where a specific cultural procedure is employed, such as the Löffler blood-serum medium for B. diphtheriæ which favors the growth of B. diphtheriæ more than the associated oral bacteria. The fourth is the examination of the feces for B. typhosus, paratyphosus, etc., which have no distinctive morphology to separate them from the normally occurring colon varieties.

The staining methods to be employed for the direct spread depend on the source of the material and what one expects to find. Depending on this, we commonly employ methylene blue or alkaline methylene blue or the purer azur stains, (such as Giemsa), the Gram stain, the acid-fast stain, the capsule stain or two or more of these staining methods.

Microbe Cultivation Methods.—The medium to be selected and the method of use will depend on the clinical picture, the source of the material, what may be expected from such sources, the help that is given by the direct smear and a knowledge of the cultivability of the microorganism likely to be present.

Another item to consider is the question of purity of the material.

Probably the best general medium is blood-agar. If possible, pour-plates and streak-plates should be made; if only one can be made, the latter by preference. This medium is not only especially satisfactory for growth, but also gives helpful characteristics of growth as well. In special instances serum-agar may be preferable because of its transparency. Either of these media may be used in tubes (slants), but the obtaining of separate colonies is not so easy as with plates, and the chances of overgrowth of one type by another is therefore greater. In the absence of more suitable media Löffler's blood-serum medium may be employed. For specialized procedures, such as B. typhosus from feces, direct cultivation of tubercle bacilli from sputum, etc., see special chapters. In the case of cultures from the sputum the following procedure is employed:

Sputum Washing.—Some of the associated bacteria found in the expectoration come from the diseased areas of the lungs, while others are merely added to the sputum as it passes through the mouth or are developed after gathering. To endeavor to separate the one from the other we wash the sputum. The first essential is that the material is to be washed within a few minutes, and certainly within an hour after being expectorated. If a longer time is allowed to intervene, the bacteria from the mouth may penetrate into the interior of the mucus, and thus appear as if they came from the lungs. Sputum treated twenty-four hours after its expectoration is useless for examining for anything except the tubercle bacillus. A rough method is to pour some of the specimen of sputum to be examined into a convenient receptacle containing sterile water, and withdraw, by means of a sterilized platinum wire, one of the cheesy masses or thick "balls" of mucus. Pass this mass five times through sterile water in a dish; repeat the operation in fresh water in a second and third dish. Spread what remains of the mass on cover-glasses and make smear preparation; stain and examine. With another washed mass inoculate ascitic bouillon in tubes and agar in plates.

When we wish to exclude more thoroughly the mouth bacteria, a lump of the

sputum raised by a natural cough is seized by the forceps and transferred to a bottle of sterile water and thoroughly shaken; it is then removed to a second bottle (of bouillon) and again thoroughly shaken. From this it is passed in the same way through four other bottles of bouillon. A portion of the mass is now smeared over cover-glasses, and the rest inoculated in suitable media, such as agar in Petri dishes, and ascitic fluid bouillon in tubes. If desired, the bacteria washed off in the different washings are allowed to develop.

Blood Cultures.—For the detection of the bacteria causing septicemia we are met with the difficulty that there are apt to be comparatively few organisms. The numbers present will vary in proportion to the severity of the infection and will be most numerous just before death. As there may be only a few microorganisms per cubic centimeter of blood or even much less in milder infections, a considerable amount of blood may be needed to obtain a positive culture. From 5 to 20 c.c. of blood is taken from the median basilic vein or any vein which can be entered. This is done by needle and syringe. The vein should be extended by pressure on the side of return flow. The most rigid asepsis is required throughout the procedure.

The collected blood may be emptied into a sterile tube containing citrate or oxalate and brought to the laboratory for culture or it may be added to the appropriate media at the bedside.

As a general rule the blood is added to agar (1 c.c. of blood to 10 c.c. of melted agar cooled to 40° C.) and pour-plates made, and like quantities or more are added to small flasks or bottles of broth. The agar plates give an indication of the number of microörganisms per cubic centimeter. The broth may in some instances give positive finding where the agar fails. A good quality of agar or broth containing the vitamin factors is in general the medium of choice. Media should be tested as to their suitability for growth of the more tenuous bacteria before use. It may require several days for growth to develop in some tenuous types. As a rule blood cultures should be held for a week. The more specialized procedures, such as bile medium for B. typhosus, will be noted in the later chapters.[1]

Material for Serological Tests.—A drop of blood from finger or ear collected on a clean slide and allowed to dry is the commonest method of collecting material for agglutination tests. Blood may be collected in Wright capsules and the serum thus obtained may be preferred. For certain tests (complement-fixation) a greater quantity of serum is desired and a tube with needle for vein punctures is used. Cerebrospinal fluid and other transudate fluids are used in special tests.

Blister fluid can be used for the agglutination test. A blister can be raised quickly by placing a piece of blotting paper moistened with a little strong ammonia on the skin and covering with a watch-glass, or one may be more slowly formed by a cantharides plaster.

Obtaining Material From Autopsy Material.—From the dead body culture material should be removed at the first possible moment after death, both because of increase of the end invaders, the secondary invaders, and of the postmortem invasion of the tissue organs by intestinal bacteria, with resultant overgrowth masking the primary microörganism.

The same precautions for avoiding contamination should be observed here as in obtaining material from the living. Thus, if we wish to obtain material from an abscess of the liver, where the organ lies in a peritoneal cavity infected with microbes, one must first absolutely sterilize the surface of the liver by pressing on it the blade of a hot-iron spatula or preferably a broad flat piece of iron mounted so as to prevent heat transmission to the handle. After surface sterilization the abscess is opened with sterile knife and the pus obtained. The same technique is used for other organs. Intestinal contents are best collected by

[1] Special vacuum tubes (with or without media) are obtainable. The needle is introduced into the vein, the closure holding the vacuum broken and the blood thus sucked into the tube.

tying and cutting out sections of the gut and transporting these to the bacteriological laboratories.

As the heart blood may be invaded postmortem, blood cultures should be made from a peripheral vein in case the autopsy has been delayed.

Smears, cultures and possibly animal inoculation are used depending on the same criteria as discussed above (material from living subject). The postmortem findings serve as further indications.

The cut sections may be stained for bacteria. In some suspected rabies, typhus etc., the examination for special bodies, Negri, Rickettsia etc., will be indicated.

The following summary with Gram stain reactions gives the most important indicative facts as regard source and history.

Gram-negative bacilli.	Coli group.	Most frequently from intestinal tract.	Most frequently found, and some indication of their presence in history.
	Typhosus group.		
	Dysenteria group.		
	Proteus group.		
	Capsulatus group.		
	Pyocyaneus.		
	Influenza group.	Most frequently from chest contents.	
	Pertussis.		
	Fusiformis.		
	Malleus.		Less frequently found, and generally a marked indication of their presence in history.
	Pasteurella group.		
	Alkaligenes (Brucella) group.		
Gram-negative cocci.	Micrococcus (Neisseria) meningitidis.		Generally marked indication of their presence in history.
	Micrococcus (Neisseria) catarrhalis.		
	Micrococcus (Neisseria) gonorrhoeæ.		
Gram-negative spirilla.	Choleræ and allied forms.	Marked indication of presence of first form in history.	
	Mouth spirals.	Unimportant, unless indicated in history, when Tr. pallidum or Sp. recurrentis, respectively, should be looked for.	
	Tr. pallidum.		
	Sp. recurrentis.		

Old forms of any of the Gram-positive or Gram-amphophile organisms.

If only *Gram-positive* organisms are demonstrated, the material may contain one or more of the following:

Gram-positive bacilli.	Diphtheriæ group.	Generally marked indication of their presence in history
	Tetani (not often demonstrated in smears from lesion) and other Clostridium species.	
	Acid-fast bacilli (tubercle bacilli, leprosy bacilli).	
	B. anthracis.	
Gram-positive cocci.	Staphylococcus group.	Some indication of their presence in history.
	Streptococcus group (including pneumococcus).	
Gram-positive spirilla.	None.	
Higher bacteria.	Leptotrichia.	
	Actinomyces (most species).	

Yeasts and moulds, certain forms.

If organisms partially stained by Gram's method are demonstrated, the material may contain one or more of the following:

Amphophile.
- Moulds.
- Yeasts.
- Protozoa.
- Certain of the Clostridium species.
- Slightly known cocci and bacilli.

Older forms of any of the Gram-positive organisms.

For *regional distribution* of microörganisms as well as other points of differentiation see Table on Identification, etc.

The question whether animals shall be employed will depend on a consideration of the same factors as given above.

CHAPTER VII.

THE RELATIONSHIP OF MICROÖRGANISMS TO DISEASE AND THE RESISTANCE OF THE HOST TO MICROBAL INFECTION.[1]

In preceding chapters we have considered the growth of microorgans, for the most part, on dead organic substances. Now we must consider their growth on or in the living host and the effect of their growth and their products upon the health of the host. Although the living body serves as a food mass for the growth of certain microörganisms, it is not a passive source of food supply. Substances in the cells of the body, as well as in the blood and lymph and other secretions, are relatively antagonistic to the growth of microörganisms. Under the influence of health or response to infection these germicidal substances may increase through cell activities, while the efforts of strain, hunger and acute poisoning may cause them to diminish.

Parasitic and Pathogenic Microörganisms.—Certain microörganisms have become adapted to growth upon the physiological exterior of the body: that is, upon the mucous membranes of the upper respiratory tract, of the alimentary canal and upon the skin. Whatever antagonistic action the local secretion may possess is compensated, partly at least, by adaptation of the microörganisms. The adaptability of microörganisms to the sites mentioned is primarily dependent upon the temperature, food and reaction (acidity or alkalinity) requirements of the microörganisms. As far as we know this multiplication is usually without ill-effect upon the health of the host. Under certain conditions, irritation of the mucous membranes may result, and to some extent small numbers of the microörganisms may find their way into the deeper tissues or in the blood and lymph only, to be destroyed by the defensive mechanism of the body. Such microörganisms, as long as they do not produce inflammation of the mucous membranes, or invade, multiply and injure the deeper tissues or organs, exist for these individuals as harmless

[1] For more detailed discussion and bibliography of the subject matter of this and the following chapters, reference is made to the following:

I. G. Adami: Principles of Pathology, vol. i. H. Zinsser: Infection and Resistance. J. A. Kolmer: Infection, Immunity and Specific Therapy. P. Ehrlich: Studies in Immunity (translated by C. Bolduan). J. Bordet: Studies in Immunity (translated by F. P. Gay). E. Metchnikoff: Immunity in Infective Diseases (translated by F. G. Binnie). H. Zinsser, J. G. Hopkins and R. Ottenberg: Laboratory Course in Serum Study. H. G. Wells: Chemical Pathology; also, The Chemical Aspects of Immunity. S. Arrhenius: Immunochemistry. H. Bechold: Colloids in Biology and Medicine (translated by J. G. M. Bullowa). Loeb, J.: Proteins and the Theory of Colloidal Behavior. Peterson, W. F.: Protein Therapy and Non-specific Resistance. Also special sections in Kolle and Wassermann: Handbuch der Pathogenen Microörganismen. See also special chapters by various authors in The Newer Knowledge of Bacteriology and Immunology, edited by E. O. Jordan and I. S. Falk.

parasites or commensals. The ability to harm the host is inherent in many of the varieties of microörganisms normally present in the areas noted, that is, they are potentially pathogenic or able to produce disease if the host's resistance is lowered or if transferred to others who are more susceptible. Certain types of microörganisms which are parasitic but not generally present, have pathogenic characteristics much more highly developed. These types are, as a rule, the etiological agents in the more specific or more highly differentiated types of disease. Because their pathogenicity is more highly developed or they find no immunity, they cause disease with greater regularity when they reach a host. A varying proportion, however, because of the resistance of the host fail to develop disease, but the microörganism may multiply on the physiological exterior of the body existing under these conditions as harmless parasites. (See below under Carriers.) Parasitism may be beneficial to the host in that the bacterial products may increase his resistance to invasion or stimulate the production of substances which will neutralize bacterial poisons.

Disease-producing Power or Pathogenicity.—The disease-producing power or virulence of microörganisms is susceptible to variation. Virulence is the term we employ to indicate the relative infectious ability of a microörganism or its capacity to overcome the protective mechanism of the host. Virulence is fundamentally the relative ability of a microorganism to infect. Bacteria are broadly divisible into those which possess a marked ability to invade the interior of the body and those whose site of multiplication is more limited to the tissues covering the physiological exterior. In each the disease effects are due principally to their toxic products. Until recently it was thought that the virulence of a relatively less invasive variety, such as B. diphtheriæ, was largely dependent on the toxin-producing capacity, even though the seriousness of an infection was not necessarily directly proportionate to the toxin-producing ability. We now believe, however, that the ability to infect may be lowered or lost although the toxin-producing capacity be fully retained.

The tendency to limit the term virulence to the more definitely marked examples of invasive ability and to denote by toxicity the disease-producing properties of the less invasive exotoxin producing varieties therefore is not justified.

It has been generally believed that under natural conditions the virulence of a microörganism tends to be increased until it reaches its limit by the rapid transfer from diseased hosts to new hosts which develop disease, that the virulence is highest as it leaves the case of actual disease, that the virulence tends to fall if it persists after recovery as a simple parasite or passes to new hosts, where it fails to infect, but the new hosts become carriers. This is apparently true of B. diphtheriæ. Thus in New York City 200 cases of diphtheria may be reported in a week but observations indicate that at the same time there exist from 20,000 to 50,000 B. diphtheriæ carriers. Investigations show that at least 50 per cent. of the actual cases are due to

contact with other cases. This indicates that less than 50 per cent. are due to carriers which are at least 100 times as numerous as the cases. Investigations at the Johns Hopkins University[1] and in New York by Park on families in which a known B. diphtheriæ carrier exists showed that not more than 1 per cent. of such families had an actual case of diphtheria. Flexner, Webster[2] and their associates have carried out extensive epidemiological studies on colonies of mice and other animals. They believe that their results show a relative fixity of virulence, and that other factors, such as individual susceptibility and dosage, are the deciding factors in infection. They did find, however, on the decline of the outbreaks in the mice that the fresh mice added were not as liable to contract disease from carriers as were mice added at the beginning of the outbreak. Topley and his associates,[3] who have carried out similar observation, believe that there is an indication of change of virulence.

In the laboratory the invasive capacity and to a much less extent the toxicity of some microörganisms can be easily modified. Invasive virulence may be decreased by cultivation upon artificial culture media, by exposure to high temperature, which, however, is not sufficient to kill, by desiccation, by exposure to light, by the action of small amounts of antiseptic substances, and so on. Invasive virulence may be increased by the addition of body fluids to culture media, by passage through successive animals of the same species or by cultivation in collodion sacs placed in the tissues or cavities of living animals. An increased virulence for one species of animal is not necessarily associated with an increase for other species. In some cases the increase for one is associated with a lowering for another.

Different cultures of one and the same type of bacterium vary widely in their ability to produce toxin, but the exhibition of the maximum ability of the individual strains rests ordinarily upon the suitability of cultural conditions.

The individuals in a culture when separated and cultivated as strains will differ in their ability to produce toxin.[4] Colony variants have been found which yield cultures that differ in their toxin-producing capacity.[5] The culture as a whole, shows, however, a tendency to remain relatively constant. Thus the culture of B. diphtheriæ that we employ for the production of diphtheria toxin has been in cultivation for thirty-four years.

Northrup and De Kruiff and others have observed differences in P. D. or "charge" (the electrical potential difference between a particle

[1] Doull, Stokes and McGinnes: Jour. Prev. Med., 1928, **2**, 191. Frost: Jour. Prev. Med., 1928, **2**, 325.

[2] Amoss: Jour. Exp. Med., 1922, **36**, 25, 45. Webster: Jour. Exp. Med., 1924, **39**, 129. Flexner: Am. Jour. Med. Sci., 1926, **121**, 69. Webster: Jour. Exp. Med., 1928, **47**, 685; 1927, **45**, 911; **46**, 887.

[3] Topley, Weir and Wilson: Jour. Hyg., 1920, **20**, 227. Topley: Lancet, 1919, ii, 1, 45, 91; Jour. Hyg., 1921, **19**, 350; 1921, **20**, 103. Topley, Greenwood, Wilson and Newbold: Jour. Hyg., 1928, **27**, 396.

[4] Crowell: Jour. Bacteriol., 1926, **11**, 26.

[5] Cowan: Brit. Jour. Exp. Path., 1927, **8**, 6.

and its menstruum) which paralleled differences in virulence. Falk[1] has recently carried out further investigations, especially with pneumococci, and believes that a high P. D. means high virulence, a low P. D., low virulence.

In the case of a toxin-producing bacterium B. diphtheriæ[2] he reports that the P. D. varies with the toxin-producing capacity and recommends the electrophoresis procedure to differentiate true or toxin-producing diphtheria bacilli, from the non-toxic diphtheria-like varieties. We doubt whether the difference is definite enough to be used as a routine method in place of the animal test.

Influence of Quantity on Infection.—If microörganisms are extremely virulent and the resistance of the host is slight, infection may result when very few or even one gains entrance to the body. When less virulent the introduction of increased numbers may compensate for the lower virulence. In the latter case infection may result if the body resources immediately available are exhausted in destroying a smaller or greater part of the microörganisms introduced, thus allowing those that survive to multiply and as the generations develop to increase gradually in virulence. Or the apparent influence of increased numbers may be partly due to the presence of a few microörganisms of somewhat higher virulence than the majority. Both factors might operate.

Mixed and Secondary Infections.—An infection may be due from the start to more than one variety of microörganism. This is most commonly observed in infections of wounds and where the skin and mucous membranes are involved, or in the extension of infection from these membranes.

Secondary infections, that is, the implantation of one or more varieties of microörganisms upon an existing infection, are common. To a large extent this is due to the lowering of tissue resistance by the toxic products of the initial organism. Secondary infection is especially common in infections of the skin and of the respiratory or intestinal tracts because of the normal presence of many varieties of bacteria or of the addition of new forms from outside contamination. These bacteria under normal conditions exist as parasites even though they may possess some degree of virulence. With the reduction of resistance or actual destruction of the skin or membranes due to inflammation one or more varieties may be able to cause secondary infection. Thus in smallpox, staphylococci invade the skin lesions caused by the smallpox virus, in intestinal infections bacilli of the colon types are commonly present as secondary agents and aid in extension of the inflammation. Complicating lesions due to extension of infection from the respiratory or intestinal tract are commonly mixed infections due to the invasion by the primary and a secondary infecting organism or at times to the invasion of two or more secondary organisms only. In fact, in many diseases the secondary

[1] Falk and Jacobson: Jour. Bacteriol., 1927, **13**, 191; Jour. Inf. Dis., 1926, **38**, 182, 188; 1925, **37**, 507.

[2] Falk, Jensen and Mills: Proc. Soc. Exp. Biol. and Med., 1928, **25**, 428. Falk, Tooney, White and Jensen: Am. Jour. Publ. Health, 1927, **17**, 714.

infection when once established may be much the more important, not only in prolonging and extending the infection but also as a cause of death. Where two or more infectious agents are present and a general blood invasion follows, as a rule, only one type will have invaded the blood stream. In the case of secondary infections the invading type is frequently the secondary organism.

Another factor in mixed or secondary infections is the influence of the microörganisms one upon the other. One may aid the other as, for example, the presence of aërobic bacteria in a wound promotes the development of the tetanus bacillus and its spores, probably by absorbing uncombined oxygen, in the presence of which B. tetani cannot multiply. One may injure the other as, for example, the pyogenic cocci in anthrax.

Protective Powers of the Host.—In the production of disease the degree of protective power of the host is no less important than the degree of pathogenicity of microörganisms. Upon the degree of development of this protective mechanism depends the resistance or immunity of the host.

Immunity or resistance to infection depends upon four general defences: (1) The mechanical barrier offered by the coverings of the physiological exterior of the body, viz., the skin and mucous membranes; (2) the mechanical and chemical action of secretions secreted by the various glands connected with the physiological exterior of the body; (3) the chemico-physical action of substances in the blood, lymph and fluids which bathe the body cells (ferments or antibodies); (4) the ability of certain cells to engulf and destroy microörganisms through the action of their ferments (phagocytosis).

Mechanical Barriers to Infection.—The skin and to a somewhat less extent the mucous membranes form very efficient barriers to infection. The thickness of the membrane and the relatively small number of glands, and the absence of crypts or pockets favor protection. Microörganisms may pass through these membranes following very slight injury. This may be so slight that the membranes are apparently intact.

In both instances no signs of inflammation may develop at the site of entry. Should the slight injury in the former instance heal, in neither case would any indication be left, as to the point of passage. The opening of the glands as well as crypts or folds offer places for the accumulation of bacteria. These may multiply and cause injury to the lining epithelium thus making a breach for invasion. The cilia of the healthy cells, sweeping the inspired microörganisms outward toward the mouth, aid in protecting bronchioles and alveoli.

Chemical and Mechanical Action of Secretions of Mucous Membranes.—These secretions when discharged on the surface aid mechanically by washing away the microörganisms and the toxic products of their action on the tissues. The secretions also restrain the growth or actually kill many varieties of microörganisms because of their constituents or to a less extent because of the presence of antibodies in the serous element of the secretions.

The saliva aids in preventing infections as is evidenced by the development of a fetid condition of the mouth when this secretion is diminished. The gastric juice is strongly bactericidal because of its content in free hydrochloric acid. In spite of this, however, many microörganisms pass through the stomach because of the protection by food particles as well as their greater resistance to the action of acid. The intestinal secretions are probably without effect on most microörganisms. The bile, however, is moderately bactericidal, this action being materially greater when the secretion is alkaline in reaction.

Ferments or Antibodies.—The ferments present in body fluids, which are the basis of the general body metabolism may act upon microorganisms destroying them and digesting them and their products. Certain substances of a ferment character known as antibodies may be present in the blood of normal individuals. These antibodies may destroy microörganisms or neutralize their poisons or toxins. Should infection occur, and be not too severe, their production is stimulated and their increase is an essential factor in the limitation and in the recovery from infection or disease, as well as (see below) in the protection of the individual from a recurrence of the same infection or disease.

Cellular Activity or Phagocytosis.—This ability to ingest and destroy microörganisms is possessed by two types of cells; the leukocytes, especially the polymorphonuclear type and the cells of the reticuloendothelial system. The activity of these cells is an essential protection of the body from infection.

Although phagocytosis is a cellular activity it is not independent of the antibodies, the latter are necessary in most instances to prepare the bacteria for ingestion. The mechanism of this phenomenon in relation to the prevention and to the limitation of infection is given in detail in a later chapter.

Immunity, Varieties and Factors Involved.—Immunity to one or more types of infection or disease is normally possessed by many individuals. This is termed natural immunity. All the factors discussed above underlie this type of resistance. Such natural immunity may be general or specific in character. The latter is evidenced by the resistance possessed by some individuals to certain specific diseases. Such special resistance depends not only upon the general factors given but also upon the possession of antibodies which are active against the specific causative organism or its toxins. This is especially striking in the natural immunity to diphtheria which is due to the presence of antibodies that can neutralize the toxins of the bacillus.

Immunity may be "acquired" either as the result of natural infection or disease or by the artificial production of a mild form of the disease or by the injection of the specific microörganism or its products. In this type of immunity the stimulation of the production of antibodies is probably the essential factor in the subsequent immunity. This stimulation may be followed not only by an increased content of antibody but also by an ability of the cells more quickly and more freely to respond to similar stimuli. The immunity to diphtheria possessed by many

has been looked upon as a natural immunity. This antitoxic immunity in a certain proportion of the immunes is probably due to the absorption of slight amounts of toxin while the immunes were carriers of B. diphtheriæ. The same cause may operate in the immunity acquired as age increases to scarlet fever.

Acquired immunity may be "active," that is, due to the individual's own response. It may be "passive" through the injection of blood or serum containing antibodies from, and produced by another individual or the transfer from mother to young.

Although the terms natural and acquired may be employed for general purposes of discussion as defined, the definitions given are not altogether satisfactory. Thus, there is an increased general and specific resistance to disease as individuals grow older without regard to recovery from any disease or other demonstrable reason for this increase. Although this immunity is natural in one sense in another it is distinctly acquired. Against certain infections, this is due probably to the stimulation resulting from the passage through the mucous membranes of small numbers of microörganisms. The apparent lack of virulence of many respiratory and intestinal bacteria in persons in thickly populated regions is frequently due to an immunity developed in this way. Against others the cause of the development of immunity is wholly unknown.

Natural immunity may be further qualified as "inherited," "racial," "species," "familial," "individual."

Susceptibility to Infection.—This is the reverse of immunity and is due to the absence or suppression of the activity of the factors which underlie immunity. Susceptibility may be general. This involves the absence or suppression of one or more or all these factors; or it may be specific, in which case there is a susceptibility to a specific disease due most probably to the absence of protective antibodies.

Susceptibility may be "natural" or "acquired" or may be "inherited," "racial," "species" or "individual."

Man as a species has an inherited susceptibility to the distinctively human diseases such as gonorrhea, typhoid fever, etc., whereas other species have an inherited immunity to these infections. Each species has a more or less distinctive susceptibility or immunity, thus the horse is susceptible to glanders but immune to tick fever, cattle are susceptible to tick fever but immune to glanders. Such types of susceptibility depend upon the adaptation of microörganisms to species peculiarities of cellular structure and metabolism. The immunity is the absence of such adaptation of the microörganisms.

Special peculiarities in relation to mutual affinity of cells and bacterial products are also a factor. Thus in the absence of such affinity, the cells cannot be harmed. An affinity may exist, but if the cell is not susceptible to injury the bacterial product is digested as though it were any inert substance. If a toxin had no affinity it would simply be eliminated. Such cellular peculiarities probably underlie the susceptibility of man to tetanus toxin and the immunity of the hen.

Racial immunity and susceptibility are shown by the suceptibility of

ordinary sheep to anthrax, whereas Algerian sheep are immune. Racial immunity or susceptibility in man is probably largely dependent upon exposure of the race to infection. The gradual elimination of the most susceptible is probably also a factor in the development of racial immunity. An example is the relative resistance of civilized races to tuberculosis whereas aboriginal races not only are more susceptible to infection but also show a higher mortality rate.

Young infants show a relative passive immunity to certain diseases such as diphtheria, measles, typhoid fever, etc. This is due to passive placental transfer of antibodies from an immune mother. Susceptibility develops later if these antibodies are exhausted and not replaced by the cells of the child. In some species the placenta is not permeable to antibodies and the immunity of the new-born depends on the passive transfer of antibodies through the colostrum milk. It was found by Smith and Little[1] that calves deprived of colostrum lacked resistance to infection by intestinal bacteria. These invade the body and various organs, and if the animal survives, may localize in the joints or kidneys. In most cases, however, a septicemia results which terminates in the death of the calves. Famulener[2] found that the colostrum was the chief agent in the transfer of antibodies to their offspring from goats immunized to red blood cells. Kuttner and Ratner,[3] in a 1923 review showed that the permeability of the placenta depends on its structure. Certain diseases are essentially diseases of childhood, as, for instance, ringworm of the scalp.

Susceptibility may be acquired for some diseases by depression of the general physiological activities of the body. This may result from overwork, overstrain or worry, inadequate rest or sleep, improper environment, lack of fresh air and sunlight, exposure to cold and wet, malnutrition due to insufficient or improper food, absence of vitamins in the diet, excesses in eating or abuse of alcohol, disease (organic or infectious) and trauma. Tuberculosis and typhoid fever are examples.

Certain types of individual susceptibility are of special interest because one attack of a disease seems to confer no lasting immunity. Two examples are pneumonia and erysipelas. However, different types of bacteria cause these diseases and in relatively early recurrence of infection it may well be that reinfection is due to a type other than that which caused the first infection and for which no immunity had developed. This is certainly true in some cases of pneumonia.

Other Factors in Infection.—The portal of entry of microörganisms, the site of election for multiplication and the bearing upon this special tissue immunity or susceptibility are important in determining whether infection will occur.

Tissue or Organ Susceptibility or Immunity.—The general facts concerning the protective coverings, the skin and mucous membranes, as well as the protection afforded by cilia and also by the various secretions of

[1] Jour. Exp. Med., 1922, **36**, 181.
[2] Jour. Inf. Dis., 1912, **10**, 332.
[3] Am. Jour. Dis. Child., 1923, **25**, 413, see for further references.

the mucous membranes have already been considered. The blood and lymph owe their protective power to antibodies and phagocytic cells and serve as the barrier to extension of bacteria from without to tissues and organs open only to infection by these routes (hematogenous or lymphatic). The tissues and organs themselves are protected by the tissue fluids (lymph) and such added phagocytic cells as they possess and by the relative ability of such cells to destroy microörganisms through substances in the cells. Although the general protection of the tissues and organs is basically similar, very different susceptibilities may be demonstrable, even to the same type of bacterium.

Thus the diphtheria bacillus may infect the mucosa of the pharynx and extend frequently to the larynx and bronchus, but only very exceptionally does it cause inflammation of the esophagus. Drinking of milk containing streptococci, derived from a case of septic sore-throat, may cause a throat infection, but no evident infection of the gastro-intestinal tract. Similar peculiarities are noted in relation to the portal of entry and the site of election.

Portal of Entry or Route of Infection.—Many microörganisms have definite portals of entry, to which they are adapted. If introduced by another mode they fail usually to cause infection or they cause at most only a transient local inflammation. Thus virulent cholera vibrios when swallowed cause the disease cholera, but injected into the tissues cause only a transient local inflammation. The typhoid bacillus likewise enters normally by the mouth, passes through the mucous membranes to the blood and causes a general infection, yet the introduction into the subcutaneous tissues would probably cause only a local lesion. The tetanus bacillus when introduced into the subcutaneous tissues may cause tetanus, yet taken by mouth is harmless, in fact the bacillus frequently leads a parasitic existence in the intestinal canal. The more definitely an organism is adapted to the production of a specific clinical disease the more sharply it is limited to one mode of entry. With some bacteria the type of disease produced will depend on the mode of entry, thus the plague bacillus entering through the skin causes bubonic plague, whereas, entry by the respiratory tract gives rise to pneumonic plague. It is probable that another factor is the adaptation of a germ to certain tissues.

Site of Election: Selective Localization and Toxin Affinity.—Having passed their normal portal of entry, should they be sufficiently virulent to overcome the factors of resistance given above, microörganisms select certain areas for localization and multiplication following which infection or disease results. Localization depends on tissue and organ immunity and susceptibility and differs widely with various types of microörganisms. It depends to some extent upon the mode of entry as in plague. In some diseases the site selected is directly at the portal of entry or connected with it; thus the diphtheria bacillus in the pharynx or larynx, the dysentery bacillus in the intestine, etc. The selective localization is most striking in diseases where the microorganisms invade the blood stream to localize later. Thus the men-

ingococcus selects the meninges; the virus of poliomyelitis, the motor areas of the central nervous system; the typhoid bacillus, lymphoid tissue, etc. Evidence has been advanced as already noted that races of one and the same microörganism may differ in their selective action, thus certain strains of the spirochete of syphilis injected into rabbits appear to select the ocular tissues for localization. Certain streptococci show definite tendencies to localize at the roots of teeth or in the joints or in the heart valves. Rosenow claims that the elective affinity of an individual strain breeds nearly true, when injections of rabbits are carried out with these strains. There is undoubtedly a tendency to do this. To some extent, the "screening" out of bacteria in the capillary circulation or the lodgment of small emboli, influences localization in individual cases, especially in relation to relative organ susceptibility. Thus the tubercle bacillus may localize in any organ if the resistance be low, but localizes by preference in the lung. In the latter, it again selects by preference the apices. The latter would seem to be influenced by circulatory conditions, as depending on posture of the host, the bacillus selects the most elevated apex, that of the upper lobe in man, that of the dorsal lobe in quadrupeds.

The specific toxins of microörganisms show similar selective affinities, thus the tetanus toxin when absorbed affects chiefly the central nervous system, while the diphtheria toxin acts widely on epithelium, connective tissue and certain nerve centers and botulinus toxin affects the motor nerve endings.

Types of Infection.—"Exogenous" infection refers to the entry of microörganisms from without. "Endogenous" infection, is infection by microörganisms existing as simple parasites but which because of a temporarily lowered resistance of the host are able to initiate an infection. These two types of infection are frequently not differentiable. Infection may be local or general. The former may be at the portal of entry or remote from it, in the latter case due to hematogenous or lymphatic transfer. When an invasion of the blood stream occurs and there is no evidence of active multiplication the condition is spoken of as a "bacteriemia." This is of fairly common occurrence especially in the case of more severe local infections. When the pus-producing bacteria invade the blood and localize at different points causing new foci of infection the condition is called a "pyemia." When the blood invasion is followed by multiplication in the blood stream a "septicemia" is present. It is sometimes difficult to decide whether a case is a bacteriemia or a septicemia.

When bacteria remain localized but the body cells are being injured by the absorption of the toxins produced a "toxemia" exists. The term sapremia is used to indicate the effects of the absorption of the products of growth when saprophytic or simple parasitic bacteria grow upon diseased or injured tissues.

A "focal infection" is one which acts as a focus for the dissemination of infectious material to other parts of the body. A focal infection may be the initial point of infection or may have been originally hemato-

genous in origin. An example would be an apical abscess of the tooth, which may later be a focus for infection of the endocardium, the joints, etc.

Sources of Exogenous Infection.—As has been stated pathogenic microörganisms are usually most virulent when in or just as they leave the infected body. As a general rule, therefore, the more direct the transfer from host to host the greater is the probability of infection. For the transfer of infection and the continuance of a disease, it is necessary that microörganisms be discharged from the body. The avenues of exit will depend upon the site of localization. In the case of localized infections they will be discharged in the secretions or excretions from this area (saliva, sputum, urine, feces, etc.), or in case of abscesses in the pus from the broken-down lesion. In disseminated infections the microorganisms may be discharged to a variable extent in a number of the secretions or excretions.

Diseases which are directly transmissible are called "contagious." Upon the virulence, the amount of the virus excreted and relative and numerical susceptibility of those exposed will depend the degree of "contagiousness." Contagious diseases are less frequently transferred by contamination of inanimate objects, "fomites," such as eating utensils, clothing, bedding, contents of rooms, money, etc. The probability of transfer is directly proportionate to the time elapsing since contamination of the "fomes." Immediate transfer is the most dangerous.

Food and drinking material may be contaminated and be a serious source of infection. The probability of infection is enhanced if the material serves as a foodstuff for the further development of the contaminating agent, as, for instance, streptococci and B. typhosus in milk.

Insects may act as a mechanical means for the transfer of disease. Biting insects may be the only mode by which the virus of the disease finds an exit from the infected host as in malaria. In most instances, especially in the case of protozoan diseases, a life cycle must be completed in this "intermediate host" before the virus becomes transferable to a new host. In some instances the virus is transmitted to the insect's offspring, thus in "tick fever" of cattle it is the offspring, not the biting female that transfers the disease.

Transfer may occur by a third person acting as a mechanical agent. This is apt to occur when cleansing of the exposed skin of the third person is omitted or inadequate and the hair and clothes are not protected from contamination. Where any considerable time elapses since contact, there is a strong probability that the transfer is due to the development of the "carrier state."

Human Carriers of Disease Germs.—An individual may become a carrier following exposure to infection. This carrier condition may develop without any evidence of actual disease. In this case the individual possesses at least a slight immunity, but the infectious agent becomes implanted as a simple parasite. Such a carrier is called a "normal or contact" carrier. Should disease develop in a susceptible person and recovery take place, the infectious agent may remain as a

simple parasite, the body being protected from further injury due to the immunity developed in the process of recovery of the disease. Such a carrier is a "convalescent carrier." Frequently the "convalescent carrier" state is due to the persistence of chronic foci of inflammation in the crypts of the tonsils, in the gall-bladder or elsewhere. Because of the situation of these foci and the character of the local secretions the infectious agent is protected from antibody activity. These lesions remain limited and the body is protected from reinvasion or reinfection, due to the protective mechanism which developed and caused recovery from the disease.

Carriers, both normal and convalescent, may be transient, temporary or chronic. The last is more likely to occur in convalescent carriers. Contact carriers although usually transient or temporary may become chronic. There is reason to believe that the infectious agent, B. typhosus, for instance, may invade the body, however, failing to develop appreciable disease because of the host's immunity, and produce the same localized lesion in the biliary system, as in a case of disease.

The carrier, therefore, is in some respects a more dangerous source of infection than is the diseased individual. He not only excretes the infectious agent, but may also because of his apparently healthy condition be unsuspected. Equally insidious sources of infection are cases of disease with mild or negligible symptoms, the so-called "missed" or "passed" cases. Earlier in this chapter evidence was presented that the microörganism from carriers may show a lessened virulence. One inclines to the belief that such a loss of virulence does not usually occur where the carrier condition is due to the persistence of subacute or chronic lesions.

Local Effects of Infection.—Microörganisms in sufficient numbers, even though they possessed no toxic qualities, would cause a local interference, as would other foreign bodies in the tissues. Possessed as they are, however, of toxic substances the latter is the dominant cause of local response. Various kinds of inflammation may develop, such as serous, fibrinous, hemorrhagic, catarrhal, croupous, membranous, purulent, necrotic, gangrenous or proliferative or productive. An organism may incite an inflammation that is more or less specific, in that one of these types of inflammation dominates. The same type of inflammation, however, may be caused by other species. Some produce no peculiar response. The character of the inflammatory response depends to a great extent on the site of the lesion, and depending on the site of localization, may differ considerably even with one and the same microörganism. The character and extent of the local lesion will depend on the virulence of the microörganisms, the number introduced and the local resistance of the tissues. The following are examples of the types of inflammatory response to infection by various microorganisms. Thus inflammation and serous exudation into the subcutaneous tissues follow injections of the pneumococcus or anthrax bacillus. The development of the streptococcus or pneumococcus in the endocardium or pleural cavity is followed by a serous exudation, frequently

with more or less fibrin production. The formation of pus results more especially from the streptococcus, pneumococcus and staphylococcus; but nearly all forms of bacteria, when they accumulate in one locality may produce purulent inflammation.

Catarrhal inflammation, with or without pus, follows the absorption of products of many bacteria, such as the gonococcus, pneumococcus, streptococcus and influenza bacillus, etc. The hemorrhagic exudation seen in pneumonia is usually due to the pneumococcus; it is observed also in other infections. Cell necrosis is produced frequently by the products of the diphtheria and of the typhoid bacilli and by those of other bacteria. Specific proliferative inflammation follows the localization of the products derived from the tubercle bacillus and the leprosy bacillus.

General Effects of Infection.—The systemic effects of infection will depend to some extend upon the localization or dissemination of the infection, that is, upon the disturbance of the structure and physiological function of an organ which in turn may influence the functions of one or more other organs. Such functional disturbance will depend as well upon the selective affinity of the toxin elaborated by the infecting agent.

Thus the effects on the lung of pneumococcus infection are different from those of an infection of the meninges or of a pneumococcus septicemia, although there is basically the same type of intoxication with pneumococcus products. In the case of pneumonia the most serious symptoms may not be due to the lung involvement but to the heart through mechanical interference with the circulation as well as the toxic effects of the products of infection on the heart muscle.

There is one general effect common to all infections, where sufficient bacterial products find their way to the blood and lymph, viz., fever. This may be directly due to toxins secreted by the bacteria or directly or indirectly due to the protoplasm of the microörganism. (See below Toxins and Endotoxins.) As a rule the degree of fever is an index to the degree of systemic toxemia. When, however there is an overwhelming infection or when the body cells are exhausted, little febrile response may occur or the temperature may be subnormal.

The Causation of Local and General Effects of Infection.—Although microörganisms are foreign bodies, we cannot account for the disturbance they create on the basis of mechanical injury alone. To some extent, obstruction of the capillaries by the microörganisms contributes to tissue injury or destruction. Inflammation may lead to pressure on the capillaries, on the lymphatics and on the tissue cells which may lead to further injury. Even in the extreme types of fatal septicemia such as anthrax, such factors, however, give little basis for explaining the clinical and pathological manifestations.

One of the earliest attempts to explain the toxemic effects of infection was the hypothesis that non-specific poisonous products were produced when microörganisms utilized the proteins of the host for food. Some of the cleavage products of proteins, which were termed "ptomains," formed by bacterial metabolism, were shown to be toxic. These sub-

stances are essentially putrefactive products, being the intermediate products of proteolysis, such as putrescin, cadavarin or of cleavage of lipoids, such as the lecithin derivatives neurin and muscarin. That such substances have any appreciable rôle in producing the effects of infection is very doubtful. They are produced by many bacteria which are wholly without pathogenic power, whereas highly pathogenic types commonly produce little or no ptomains. Even when ptomains are produced by a pathogenic bacterium the effect of their injection is physiologically very different from that of the toxic products produced in infection.

The gastro-intestinal symptoms of "food poisoning" have been attributed to ptomains. Intensive study has shown, however, that such enteric symptoms are more likely to be due to infection by bacilli of the paratyphoid-enteritidis group. Ptomain production and absorption may be a factor in the mixed infections of extensive areas of dead tissue as in gangrene, crushed or lacerated wounds and deep burns.

It was soon evident to earlier investigators that common poisonous substances would not explain the more or less specific effects produced by different pathogenic microörganisms. Considerable doubt has arisen as to whether the purified ptomains as isolated by the chemist are the actual poisoning products of bacterial growth and tissue necrosis or are other cleavage products resulting from chemical manipulation.

Investigation, therefore, turned in the direction of the demonstration not of general toxic substances but of specific toxic substances. Roux and Yersin, in 1889, discovered that the diphtheria bacillus when grown in broth excreted a toxin which was present in the broth even though the bacilli were removed by filtration. The poison, therefore, was "extracellular" and "soluble." In 1891 Kitasato discovered a soluble toxin in the filtrates of broth cultures of the tetanus bacillus. Both these toxins were found to reproduce the physiological effects noted in infection by their respective types of bacilli.

With these discoveries it seemed that but a short time would elapse before the demonstration of similar toxins for all pathogenic microorganisms. It was soon found, however, that most bacteria, for example the typhoid bacillus, did not produce demonstrable extracellular toxins, but that toxic substances could only be obtained by extraction or disintegration of the bacterial cell. Pfeiffer, in 1892, published such results, and called these toxins "endotoxins" in contrast to the "exotoxins," above described. At first it was thought these "endotoxins" were as specific and definite in character as the exotoxins. The knowledge about such toxic substances is extremely limited, and at best the term endotoxin serves primarily to designate such poisonous substances which are not freely excreted by microörganisms. Vaughan has advanced the idea that specific endotoxins do not exist, but that the poisonous products are the cleavage products of bacterial protoplasm which develop in the infected host. It is not improbable that some of the constituents of the microbal cell are toxic in themselves. Jobling

has shown that some of the toxic effects may be indirect, arising through interference with antienzyme activity.

The Specific Responses of the Body.—When the filtrate of a seven-day broth culture of the diphtheria bacillus is injected into animals in sublethal doses, for instance the horse, the reaction is shown mainly by the production of an antibody against the toxin which is a specific *antigen* or antibody stimulating agent. This substance is called "antitoxin." Its production is shown by the fact that increasing doses of the toxin can be injected until hundreds or thousands of what originally would have been fatal doses are given. Likewise, it can be shown that mixing the blood or serum of the immunized horse with toxin will result in neutralization of the toxin. This neutralization follows roughly a multiple scale, thus if 1 part of serum will neutralize 1 part of toxin, 1000 parts of serum will approximately neutralize 1000 parts of toxin.

If the filtrate of a seven-day broth culture of the typhoid be injected the demonstrable antibodies produced are practically all antibacterial in character, the bacterial substance in this instance being the *antigen* or antibody stimulating agent. The filtrate may be toxic, as we find that the size of successive doses can be increased, but not to any extent comparable with that noted above. After a series of injections mixing the blood or serum of the horse with the filtrate will neutralize the toxic action of the filtrate to some extent, but neutralization will not follow a multiple scale. As the amount of "endotoxin" is increased the multiple of serum required will have to be much greater. If the amount of "endotoxin" is still further increased neutralization becomes difficult or impossible, however much serum is added.

The deductions, therefore, would be that the response of the body in the attempt to recover from an infection would differ very materially as to whether the infecting organism produced or apparently did not produce an extracellular toxin. In infections by bacteria which produce no demonstrable exotoxin the response should be primarily in the production of antibacterial antibodies, which observation and study has shown to be the case.

In infections by microörganisms which definitely produce their effect by means of extracellular toxins, for example the diphtheria bacillus, the body response should be primarily the production of an antitoxin, which produced in sufficient amounts should neutralize the toxins present and the toxin as it is produced, so that the bacillus could be destroyed by the action of antibacterial antibodies and cells. As a matter of fact, this is not the essential mechanism of recovery, as is shown by the fact that about 75 per cent. of individuals remain relatively susceptible to diphtheria toxin immediately after recovery; that is, they have produced no detectable antitoxin. Several weeks later antitoxin may be demonstrable.

In scarlet fever antitoxin is quickly produced, and the disappearance of the toxic symptoms of the disease is due to this. However, the development or non-development of pyogenic effects depend on the antibacterial ability of body.

In all infections eventual recovery is essentially antibacterial in character; that is, through the specific antibacterial antibodies as well as the activity of the phagocytic cells. However, antitoxin development may be a distinct factor, as it may be assumed that the limitation of the toxemia protects the cells engaged in antibacterial activities.

Another phenomenon is intimately associated with the process of recovery as well as with the development of immunity, viz., hypersensitiveness. (See later chapter.)

After complete recovery from infection there is no demonstrable increase in the phagocytic capacity of the body cells.

Non-specific Factors.—In the preceding discussion only the specific responses of the body to specific antigens has been considered. Apparently non-specific stimuli play a definite rôle in immunity. Experimental work indicates that the injection of a bacterial vaccine may cause a slight increase in resistance to infection by a heterologous bacterium. There is a suggestion, at least, that the congestion in large cities leads to an increased resistance not only to those infectious agents to which the inhabitants are exposed but also to other infectious agents. Non-specific stimuli may increase the specific antibody content of the blood. Thus Collins[1] found that the agglutinin content for certain bacteria was increased by the injection of organized ferments such as yeasts or by unorganized ferments as diastase, pancreatin and invertin, likewise, by the injection of nuclein, lecithin proteoses and inorganic salts containing sulphur and phosphorus. Madsen[2] found that other antibodies as well as the agglutinins were increased by the injection of chemical substances. Injection of manganese salt for example, will increase the antitoxin content of the blood. The injection of non-specific substances is employed as a therapeutic agent.[3] In the attempt to explain the beneficial results thus obtained it has been suggested that one of the factors is that the non-specific agent stimulates the release of so-called sessile antibodies, that is, antibodies already formed in the cells. The non-specific agent may also make the tissues more permeable to antibodies present in the blood.

Response of Microörganism to Protective Mechanism of the Host.—Welch suggested that during infection the microörganism was developing further means of combating the protective agencies of the host, possibly antisubstances against the antibodies of the host. The enhanced virulence of microörganisms fresh from an infected host or the enhancement through rapid passage from host to host is apparently an adaptation to the resisting capacity of the host. Highly virulent bacteria may be highly resistant to antibody action as well as to phagocytosis. The mechanism of this resistance is not explained. The capsule in some instances has some bearing on resistance. A similar adaptation is seen in the use of arsenic compounds in certain protozoan infections. The protozoa may develop a resistance or "fastness" to the arsenical

[1] Jour. Exp. Med., 1908, **10**, 529.
[2] Jour. State Med., 1923, **31**, 51.
[3] Protein Therapy and Non-specific Resistance, Macmillan, 1922.

agents. Striking examples of adaptation may be seen in the intensive immunization of animals, thus we have observed the development of septicemia in horses (pneumococcus, paratyphoid bacilli, etc.) in spite of the presence of very large amounts of circulating antibody.

Summary.—Natural resistance to invasion by bacteria is due to some extent to the protective coverings of the physiological exterior of the body as well as to the protective action of the secretions which are discharged on these surfaces. Natural resistance or immunity to infection may exist because of the possession of antibodies as well as because of the activities of the phagocytic cells. Such antibacterial immunity operates also against such bacteria as produce extracellular toxins. Thus resistance to infection by B. diphtheriæ may exist in the absence of an antitoxic immunity. A natural immunity may be specific. Infection causes a more or less marked increase is antibacterial antibodies which apparently is a factor in the recovery from disease. Phagocytosis likewise is an important factor in such recovery. An acquired immunity, through infection or artificial inoculation by microörganisms which do not produce an extracellular toxin, is probably also dependent to a large extent on the stimulus to the development of specific antibacterial antibodies. However, certain forms of immunity may depend on the mobilization of phagocytic cells. Antitoxic immunity may develop slowly, and is not the essential factor in recovery from infection. The antitoxic immunity which may exist in the absence of previous disease is apparently acquired. Proper modes of inoculation will cause the development of an antitoxic immunity in practically all persons not possessing it. Non-specific stimuli are probably factors in the development of immunity. The infecting microörganism is not passive to the activities of the host.

Theories Concerning the Production and Action of Antibodies.—It was soon evident to early investigators especially in the light of the large amounts of antibodies that might be produced in response to the injection of small amounts of toxin, that the antibodies were secreted by the body cells and were not due to a conversion of bacterial products.

On this basis Ehrlich elaborated his well-known "side-chain theory." This theory was first advanced in the attempt to explain the nutritive process of cells. He conceived the cell as consisting of a complex central chemical nucleus of relatively constant structure to which the cell owed its peculiar functional character. Attached to this are many chemical "side chains" which because of their affinities can take the appropriate food molecules from the body fluids. After such combination the food molecule is assimilated and incorporated in the central nucleus. This conception places the nutritive activities on a purely chemical basis. When infection occurs the toxins or other products combine with the cell by the same mechanism if there is an affinity between any of the side chains and the molecules of toxin or other microbal product. These products may find suitable side-chain, "receptors or haptines," in many varieties of cells or in only the cells of certain tissues or organs. When the microbal products combine with the side chains the molecules are available not simply as foodstuffs but are actually deleterious and the side chain or receptor is destroyed; or if sufficient receptors are involved the central nucleus is injured and dies. When the nucleus lives the receptors are regenerated. In this regeneration, however, the conception of overproduction as advanced by Weigert comes into play: that

is, the injury not only stimulates the regeneration of the destroyed receptors but an excess of these receptors. The cell possessed of more receptors than necessary for its nutritive process casts the excess off into the body fluids, these cast off receptors now constituting free antibodies. It is conceivable that if the stimuli

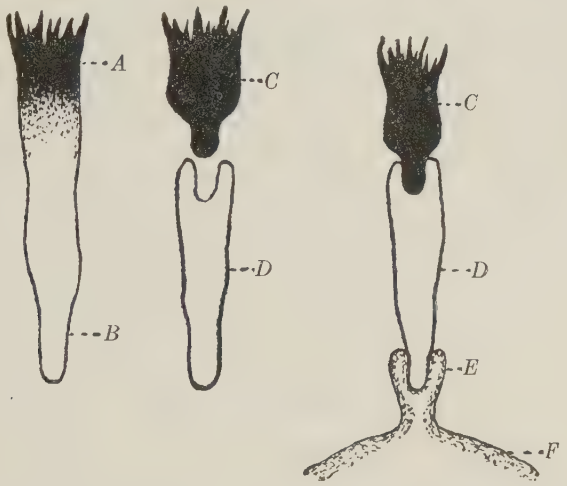


FIG. 73.—Graphic representation of receptors of the first and third orders and of complement as conceived by Ehrlich: *A*, toxophorous group of toxin; *B*, haptophorous group; *C*, complement; *D*, intermediary or immune body; *E*, foreign cell receptor; *F*, part of cell. *A B* unites directly with *E; C* unites by means of *D*.

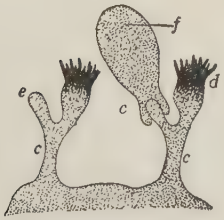


FIG. 74.—Receptors of the second order. Here *e* represents the haptophore group, and *d* the zymophore group of the receptor, *f* being the food molecule with which this receptor combines. Such receptors are possessed by agglutinins and precipitins. It is to be noted that the zymophore group is an integral part of the receptor.

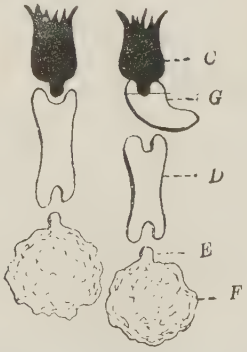


FIG. 75.— Graphic representation of amboceptors or receptors of the third order and of complement, showing on left the immune body uniting complement to foreign cell and on right the action of anti-complement, binding complement and so preventing its union with the amboceptor (see Fig. 68). *C*, complement; *D*, immune body; *E*, receptor; *F*, foreign cell; *G*, anti-complement.

to the cells were continued by the repeated partial destruction of receptors, that large amounts of antibodies would be formed in this way. The theory thus explains the general specific character of the antibodies as only those cell receptors are overproduced which have an affinity for the products of the infecting

organism. The very bodies that when connected to the cell "sessile" made possible the poisoning of the cell, constitute the antibodies when free.

These antibodies, therefore, having an affinity for microbal products will chemically combine with and neutralize them.

This theory was applied to the origin and action of the simpler antibody, antitoxin, and then elaborated to include the more complex antibacterial antibodies. Graphic representations of the theoretical structure, affinities and mode of action, according to Ehrlich, of the "antigens" (stimuli to antibody production) and antibodies are given here. This theory is, today, largely of historical interest. It had an enormous influence in the development of the subject as it

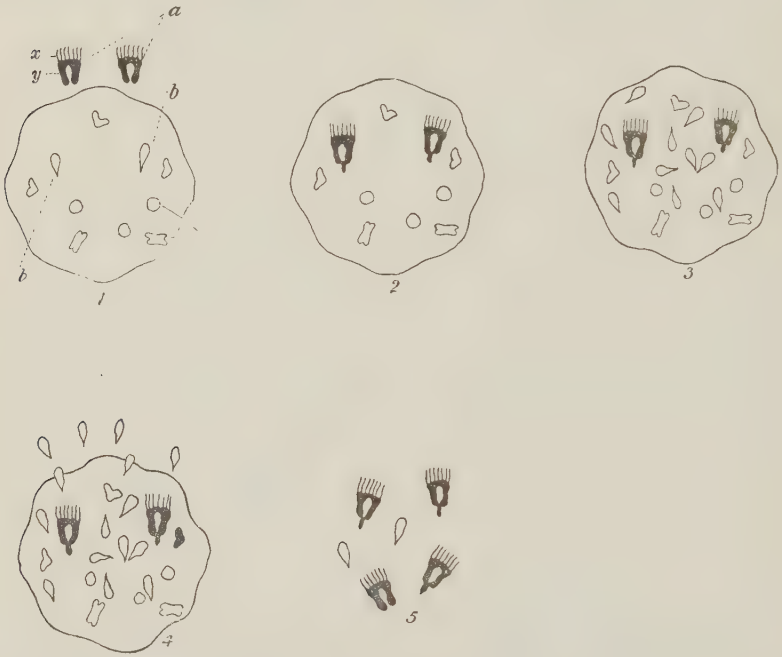


FIG. 76.—Graphic representation of Ehrlich's theory of the production of antitoxin and the neutralization of toxin: *a*, diphtheria toxin molecule; *x*, toxophore atom group; *y*, haptophore or combining group; *b*, cell receptors with affinity for diphtheria toxin; *c*, other cell receptors. 1, cell with its receptors—outside of cell, free toxin molecules; 2, toxin molecules combined with the cell receptors having affinity for diphtheria toxin; 3, after three days, showing multiplication of cell receptors similar to those combined with toxin, others not increased; 4, after four days, excess of toxin receptors cast off in the blood; 5, toxin molecules being neutralized by combining with free receptors in blood into which antitoxin had been transferred.

stimulated investigations in the attempts to sustain it and to disprove it. It does not take into consideration the later developments of physical chemistry. It has impressed on the subject a nomenclature which is in almost general use in spite of the fact that the connotation of these terms is no longer accepted. Largely through the work of Bordet and others who were stimulated by his ideas it has been shown that apparently antibody action is in most instances due to the combination of antibody with the bacterium or its substance and the evident result is due to a third factor acting on this new complex. The different types of antibodies and the hypotheses as to their action are considered more in detail in the succeeding chapters.

Origin of Antibodies.—In spite of the great numbers of experimental observations no certain results have been obtained. The spleen and the tissue phagocytes seem to have a greater capacity than any other organs or tissues. There are indications, however, that antibody formation may be a function of nearly all cells to some degree.

"Unitarian" View of Antibacterial Antibodies.—In the chapters that follow the different phenomena due to antimicrobal antibodies are considered separately. This gives the impression that agglutinins, precipitins, amboceptor and opsonins are separate and distinct antibodies. There is another view that these antibodies are not different, but that the apparent difference is only one of reaction, the character of which is determined by the physical condition of the antigen, the additive action of other factors, such as complement, etc., and the conditions under which the reaction occurs. This "unitarian" view has been advanced by several investigators. Zinsser[1] has reviewed the arguments in favor of the unitarian idea.

[1] Jour. Immunol., 1921, **6**, 289. Neill, Fleming and Gaspari: Jour. Exp. Med., November, 1927.

CHAPTER VIII.

EXOTOXIN AND ANTITOXIN.

As has been stated, microbal antigens, that is, the substances stimulating the production of microbal antibodies, can be divided into two classes: (1) The extracellular products, among which we have the exotoxins, and (2) the endocellular substance. The first stimulate the production of antitoxins, the second stimulate the production of antimicrobal antibodies, viz., agglutinins, precipitins, opsonins, bacteriolytic, bactericidal or other microbicidal substances. Each of these varieties of antibodies, as we shall see, can be produced by the injection of other substances than microörganisms or their products.

Exotoxins.—The production of an extracellular toxin is apparently possessed by only a limited number of microörganisms. The chief examples are diphtheria, tetanus, botulinus and streptococcus toxins. The first three of these toxins fulfil the criteria which had been believed for years to be the essential characteristics of a true exotoxin: (1) The production of symptoms of poisoning, however large the dose, only after a period of incubation; (2) in appropriate doses they stimulate the production of an antitoxin which has the capacity of neutralizing the toxin; (3) lability to heat and chemicals. Some exotoxins may not conform in all respects with this definition. The toxin produced by hemolytic streptococci is relatively very resistant to heat. Furthermore, the toxins of the pathogenic aërobes B. welchii[1] and Vibrion septique,[2] produce almost immediate symptoms in animals, even though a small dose be injected intravenously. In contrast to this 100,000 fatal doses of tetanus toxin injected intravenously in a rabbit will kill only after five to six hours. Therefore, the only character common to all extracellular toxins is the ability to stimulate the production of antitoxin, or most simply defined, toxins are antigenic poisons (Coca). Exotoxins have been described for B. dysenteriæ,[3] B. chauvii, B. typhosus and Vibrio choleræ, B. influenzæ and the staphylococcus.[4] The streptolysin, streptoleukocidin,[5] staphylolysin[6] and staphyloleukocidin may all be exotoxins.

Nature and Structure of Toxins.—They are products of bacterial growth and are specific. Walbum[7] has advanced evidence that the toxin as such

[1] Bull and Pritchett: Jour. Exp. Med., 1917, **26**, 119. DeKruif, Adams and Ireland: Jour. Infect. Dis., 1917, **21**, 580.
[2] Robertson: Jour. Path. and Bact., 1920, **23**, 153.
[3] Olitsky and Kligler: Jour. Exp. Med., 1920, **31**, 19.
[4] Parker and Banzhaf: Jour. Immunol., 1927, **13**, 25.
[5] Nakayama: Jour. Infect. Dis., 1920, **27**, 86.
[6] Orcutt and Howe: Jour. Exp. Med., 1922, **35**, 409.
[7] Comptes Rendus de Soc. Biol., 1922, **87**, 1224.

is not a secretory product of bacteria but that what might be termed a "protoxin" stage exists. Possibly the phenomena he observed might be taken to indicate that a "toxinase" ferment[1] was produced and that this acting on the meat extractives and peptone gives rise to the toxin. Whether this be definitely established or not we are still justified in using the term exotoxin to designate the poisonous product. Chemically toxins are seemingly protein in character. This does not exclude the possibility that the toxic element itself may not be separable, by present methods from associated protein matter. Toxins are soluble, non-crystallizable, do not dialyze except through very thin membranes. Proteolytic ferments destroy toxin. Many are very labile, being sensitive to light, free oxygen or oxidizing agents and to heat; diphtheria and tetanus toxin is destroyed by heating to 60° C. for one-half hour; even heating at 56° C. for one-half hour destroys 95 per cent. Streptococcus toxin is very resistant. Toxins are precipitated by ammonium sulphate, alcohol and nucleic acid. Ehrlich believed that the toxin molecule is composed of two essential parts: a haptophore portion, which has an affinity for the cell receptor, and a toxophore group, which causes the toxic effects (see Chapter VII). He believed that the deterioration of toxins, under ordinary conditions of storage, was primarily due to the deterioration of the more labile toxophore portion. The same type of deterioration is produced when the toxic action is destroyed by adding iodine, formaline, oleates, gold chloride, etc. Ehrlich called this residual haptophore portion a "toxoid." Theoretically, therefore, the toxoid should retain its ability to combine with cell receptors, but being no longer toxic or capable of causing irritation there should be no response in antitoxin production. This is not the case, as "toxoids" are antigenic. To explain certain observations in standardizing toxin and antitoxin (see below), Ehrlich elaborated his theory by assuming that subvarieties of toxin and toxoids existed as manifested by differences in avidity for the cell receptor or for antitoxin. To explain the later paralysis of diphtheria toxemia, he assumed the existence of a special toxic fraction which he called toxon. Late paralysis may develop in guinea-pigs in spite of antitoxin sufficient to prevent acute toxemia and death. Ehrlich assumed, therefore, a low affinity between toxon and antitoxin. If the amount of antitoxin is sufficiently large paralysis does not develop.

This conception implies a purely chemical nature in toxin. This seems to be sustained when one considers that the amount of injury done by toxin is comparable to the amount injected. On the other hand, the activity of infinitesimal doses in causing tissue injury or death or the disproportionate amount of antitoxin produced in response to toxin injection suggests the possibility that the action of toxin is not purely chemical but more allied to the action of ferments.

A broth culture of tetanus may be so toxic that 1 c.c. will suffice to kill 50,000 to 75,000 guinea-pigs. Yet the major part of this amount

[1] Madsen: Jour. State Med., 1923, **31**, 51.

of broth culture is water and meat extractives and peptone, so that the actual amount of toxin which will suffice to kill one pig is exceedingly minute. Very small amounts of toxin will stimulate the production of very large amounts of antitoxin in a horse which has been under immunization for some time.

Against the enzyme nature of toxin some have advanced the facts that toxin when introduced into the body, no matter how large the amount, quickly disappears from the body fluids due to combination with the cells for which it has an affinity. This combination is very firm and is dissociated, if at all, only with great difficulty, indicating a chemical union. In the case of tetanus toxin the lipoids of the nerve cells have a very marked influence on toxin-cell combination, indicating again that physical phenomena at least underlie the union. (See also under Combination of Toxin and Antitoxin.)

Methods of Precipitating Exotoxins.—Ammonium sulphate crystals are added to the fluid containing the toxin until it is saturated. A large excess of ammonium sulphate crystals is then added and the whole kept at about 37° C. for twelve to eighteen hours. The toxin is thrown out of solution along with the albumoses and peptones and rises to the surface. This is skimmed off and dried in a vacuum or in an exsiccator containing strong sulphuric acid. The dried powder is placed in vacuum tubes and stored in the dark. This is the method of preserving "standard" tetanus toxin. Banzhaf has obtained a diphtheria toxin that is practically free from the meat extractives in the broth, but not from the autolyzed diphtheria bacillus substances. He adds alcohol up to 65 per cent. to the slightly acidified toxin broth. The small flocculent precipitate that is formed, after standing about one hour, is filtered off and dried in vacuum. This dried toxin does not deteriorate. One gram of the powder contains 40,000 fatal doses, whereas the original toxin broth contain 5000 fatal doses per gram of solids Ammonium sulphate precipitation has been advocated as a practical method of concentrating the toxins of the pathogenic anaërobes, such as B. welchii. As these produce only relatively weak toxins, such concentration permits of the injection for immunization of smaller volume and less of the extravenous culture medium. Bacteria which secrete an exotoxin may, like other bacteria, possess endocellular substances which are poisonous. These substances, through autolysis, are present in filtered broth cultures and no one has succeeded in separating them from the true exotoxin. They constitute, as a rule, so little of the total toxicity of cultures that their effect can be ignored.

Absorption of Exotoxins.—They are absorbed with greater rapidity than are antitoxins. In a disease such as tetanus or gas bacillus infection the toxin is absorbed from the local area. In the case of diphtheria and dysentery it is probable that toxin is not absorbed until the local inflammation has led to destruction of the mucous membrane. Botulinus toxin, however, is apparently absorbed through the uninjured mucous membrane of the intestinal tract. This toxin is not destroyed

by the acid of the gastric juice. Bronfenbrenner[1] claims that acidification may actually increase the potency of this toxin. This has not been confirmed by others.[2]

Special Affinities of Exotoxins.—Different toxins cause their major symptoms by selective action on certain tissues or organs. Thus diphtheria toxin affects the vascular nerve centers and causes degeneration in the heart muscles, liver and kidneys. A specific action on the suprarenals is regularly noticed in guinea-pigs. The glands are enlarged and congested and microscopically show a degeneration or disappearance of the chromatin substances and of the medullary cells.

Tetanus toxin, according to Ehrlich, is composed of two substances: tetanospasmin, which affects the nerve cells, and tetanolysin, destructive for red blood cells. The affinity of tetanus toxin both for the nerve cells and for the red cells is apparently related to the lipoidal content of these cells.

The rash and early symptoms of scarlet fever are due to the action of the streptococcus toxin.

The toxin of B. dysenteriæ (Shiga) affects the intestinal mucosa during natural infection. When the toxin is intravenously injected into animals a similar effect is produced.

The more characteristic symptoms of botulism-poisoning are due to paralysis of the muscles supplied by the cranial nerves, those supplied by the third being first affected. This paralysis according to Edmunds and Long[3] is due to action on the nerve endings of the motor nerves.

Measurement of Toxin Action.—With toxins which cause death in animals, this is done by determining the smallest dose which will kill a susceptible animal in a certain time. To obtain comparable results a certain mode of injection and the selection of a definite animal weight must be adhered to. This dose of toxin is called the "minimum lethal dose," or the M. L. D. The standard for diphtheria toxin is that amount of toxin which, on the average, will kill a guinea-pig weighing 250 grams on the termination of the fourth day. The standard for tetanus toxin is the amount that will kill a guinea-pig weighing 350 grams in the same time. The unit of scarlet fever (streptococcus) toxin is the skin test dose (S. T. D.). To determine this tests are made on a group of susceptible children (20 by preference) and the average of the smallest amount which gives a reaction of 1.5 cm. is the S. T. D.[4] The injections are given intracutaneously, 0.1 c.c. of the dilutions being injected. The reaction is read at the end of twenty-four hours.

Methods of Producing Exotoxins.—See Specific Organisms.

Antitoxin, Nature and Structure.—An antitoxin is a specific antibody produced in response to an exotoxin and has the capacity of neutralizing only that exotoxin which stimulates its production.

[1] Jour. Am. Med. Assn., 1922, **78**, 1519.
[2] Geiger: Public Health Reports, 1923, **38**, 2249.
[3] Jour. Am. Med. Assn., 1923, **81**, 542.
[4] The method of deciding the size of the reaction that indicates susceptibility was reported by Dick and Dick in 1925, Jour. Am. Med. Assn., **84**, 1477.

Antitoxins have not been obtained in a pure state. When plasma or serum is fractionally precipitated, the antitoxin is precipitated with the globulin fraction. With diphtheria and tetanus antitoxin obtained from the horse all of the antitoxin precipitates with the pseudoglobulins. This indicates that the antitoxin is a globulin or more likely that it is associated with the globulin molecules. Antitoxin is a cellular product and according to Ehrlich's hypothesis consists of the overproduced side chains or receptors of the cells irritated by the toxin. He characterizes antitoxin as an "antibody of the first order" conceiving it as a simple haptophore which combines directly with the toxin molecule. (See Chapter VII.)

Antitoxins are relatively stable, deteriorating not more than 15 per cent. a year when refrigerated or 20 per cent. at room temperature. When dried and preserved *in vacuo* in the refrigerator practically no deterioration takes place over long periods of time. Heating to beyond 62° C. destroys antitoxin, the rapidity of destruction being greater the higher the temperature.

The Combination of Toxin and Antitoxin.—If Ehrlich's conceptions of a simple chemical combination were correct then toxin and antitoxin should combine in a directly multiple way: that is, if one part of antitoxin would neutralize one part of toxin then 1000 parts of antitoxin should neutralize 1000 parts of toxin, whatever the method of mixture. The combination is relatively but not absolutely multiple. If we determine the amount of antitoxin necessary to neutralize a certain amount of toxin and then again use the same quantities but add the toxin to the antitoxin in fractions, allowing considerable time to elapse between additions, the mixture will be found to be toxic.

In determining the unit value of diphtheria antitoxin according to the original von Behring definition unexpected phenomena were encountered. The unit referred to is the amount of antitoxin necessary to neutralize 100 M. L. D.'s of toxin. Different persons testing the same antitoxin obtained very different results unless the same sample of toxin was employed. Discrepant results were obtained when any considerable time elapsed between tests. Thus a freshly prepared toxin required more M. L. D.'s to neutralize a unit than an old toxin. Ehrlich investigated these facts by comparing the effects of the M. L. D., the Lo and the L+ dose of different toxins. The Lo dose refers to the amount of toxin apparently completely neutralized by one unit of antitoxin. The L+ dose refers to the amount of toxin which when mixed with one unit of antitoxin will still cause death of the guinea-pig on the fourth or fifth day. Theoretically, therefore, the Lo dose should equal 100 M. L. D.'s; the L+, 101 M. L. D.'s. This he found not to be the case, and the quantitative relationships of one to the other differed at times very widely, according to the samples of toxin employed. A toxin might give a M. L. D. dose of 0.0025 c.c., an Lo dose of 0.125 c.c. and L+ dose of 0.25, which showed that the theoretical values were not obtained. He observed that, although the toxin containing one M. L. D.

might increase greatly in volume, due to deterioration, the Lo quantity remained more constant. He studied the subject further with a method of partial neutralization. With this method he obtained results, of which the following figures are representative. In the first line no antitoxin was used, but the test of the M. L. D. content of the L+ dose toxin is inserted for comparison.

Antitoxin 0 unit + toxin L + dose = 85.0 M. L. D.
Antitoxin 0.25 unit + toxin L + dose = 60.0 M. L. D.
Antitoxin 0.80 unit + toxin L + dose = 10.0 M. L. D.
Antitoxin 0.90 unit + toxin L + dose = 3.5 M. L. D.
Antitoxin 1.00 unit + toxin L + dose = 1.0 M. L. D.

To explain these results and still retain his hypothesis of a simple quantitative combination, he predicated the production by B. diphtheriæ of two primary substances, a toxin and a toxon, both of which bind antitoxin, the latter having the weaker affinity. The toxins and probably the toxons deteriorate to secondary substances, toxoids, which again differ in their avidity for antitoxin. In the final analysis Ehrlich assumed, therefore, that two modifications of toxin existed and that the valency or combining value of a unit was 200 not 100, or that one antitoxin unit represented 200 valencies; and when mixed with an L+ dose of toxin left one toxin unit free. Theoretically, therefore, if a toxin could be produced containing only toxic or killing units the Lo dose would contain 200 M.L.D.'s and the L+ dose 201 M.L.D.'s (See below Antitoxin Unit.) As Ehrlich assumes that toxin and toxon are produced, and as production is quickly followed by deterioration, even the earliest produced toxin would never approach the theoretical value given above. As a matter of fact a toxin may have a Lo dose of over 200 for we developed at least one toxin whose valency was slightly over that amount. The fact that Ehrlich was forced to assume the existence of many different kinds of toxin makes the whole hypothesis very doubtful.

Arrhenius and Madsen attempted to explain the phenomena noted above by comparing the combination of toxin and antitoxin to that which occurs with a weak acid and weak base. In this case the reaction would be incomplete and reversible. Chemical equilibrium would follow their mixture, but the combination would be incomplete, so that free base and acid would always be present. The proportions of these three constituents would depend upon the concentration of the solutions employed according to the physical law of mass action. Likewise, the fractional addition of one to the other would change the neutralizing value of the total amount added. Arrhenius and Madsen studied the values obtained by adding boric acid in fractional amounts to ammonia and found that the rate of neutralization was closely parallel to that obtained by Ehrlich in his partial neutralization method.

It is likely that neither of these theories is valid. Bordet believes the combination of toxin and antitoxin to be an adsorption phenomenon of two colloids. He believes that when antitoxin is added to toxin it is distributed equally over the toxic molecule. As more antitoxin is added there is a proportionate reduction in the toxicity of each molecule until complete neutralization results. The reaction, therefore, is analogous to a staining operation, the dye staining all the substance to which it is added, the depth of the color being proportionate to the amount of dye

added. Maver[1] has recently reviewed the evidence in favor of the adsorption hypothesis. Falk and Maver[2] report that antitoxin is certainly and toxin is probably amphoteric, which conclusion is not in accord with earlier deductions of Field and Teague, who believed that both were electro-positive. The former suggest that antitoxic action may be due to the specific reduction of the "P. D." of the toxin with flocculation which renders the toxin inert.

Standardization of Diphtheria Antitoxin.[3]—The original definition of a diphtheria antitoxin unit was the amount which would neutralize 100 M. L. D.'s of freshly made diphtheria toxin. This still remains a good non-technical definition. Because of the phenomena already described, this definition of a unit was modified by Ehrlich, so that a unit today refers to that amount of antitoxin which when added to an L+ dose of a toxin and injected will neutralize the L+ dose of toxin to a degree that the life of an average 250-gram guinea-pig will be prolonged up to the end of the fourth day. The amount of antitoxin for a unit is chosen which suffices to protect for only four days, because it shortens the time required to make the test and gives, in fact, a more uniform result. As it is very important that the different laboratories use the same unit, the central governments should preserve under ideal conditions thoroughly tested material and distribute this to the biological plants. Today the Hygienic Laboratory supplies such a standard antitoxin by which toxin is standardized for use in antitoxin testing by the different producers of antitoxin. The following is a copy of one of our tests for determining the strength of diphtheria toxin:

Method of Standardizing Diphtheria Antitoxin.—The L+ of the standard diphtheria toxin is determined by adding to 1 unit of diphtheria antitoxin, as supplied by the Hygienic Laboratory, Washington, D. C., such an amount of diphtheria toxin that the mixture will kill a 250-gm. guinea-pig in three and a half to four days. We estimate our L+ = 0.71 c.c. so we make the following test:

	24 hrs.	48 hrs.	72 hrs.	94 hrs.
1 unit + 0.71 c.c. in G. P. 255 gm.	235	220	200	Dead.
1 unit + 0.72 c.c. G. P. 250 gm.	225	200	Dead.	

We now make our test of a new preparation of the diphtheria antitoxin. We believe it may have 1300 units per cubic centimeter. 1 c.c. of antitoxin into 100 c.c. volumetric flask; saline up to 100 c.c.; dilution = $\frac{1}{100}$.

1 c.c. of above into a small flask + 12 c.c. saline; dilution 1 c.c. = $\frac{1}{1300}$.

1 c.c. of last dilution (1 unit) + 0.71 c.c. (L+) of standard toxin into another flask—allow to stand one-quarter hour—inject subcutaneously into a 250-gm. guinea-pig. Make a similar mixture to test for 1400 units.

	24 hrs.	48 hrs.	72 hrs.	94 hrs.
Anti. prep. $\frac{1}{1300}$ + 0.71 c.c. G. P. 250 gm.	235 sl. Ind.	220 Ind.	210 Ind.	Dead
$\frac{1}{1400}$ + 0.71 c.c. G. P. 255 gm.	225	250	Dead	

[1] The Physical Chemistry of Toxin and Antitoxin, The Newer Knowledge of Bacteriology and Immunology, p. 739.

[2] Jour. Immunol., 1927, **14**, 219.

[3] For gas gangrene antitoxin see Hyg. Lab. Bull., 1920, **122**, 13. For botulinus antitoxin see Am. Jour. Public Health. 1921, **11**, 352.

Autopsy—congestion at site of injection and adrenals in both animals.

The death of the one pig at the end of the fourth day indicates that the antitoxin strength is just 1300 units per c.c.

Intracutaneous Method.—Amounts of diphtheria toxin too small to kill a guinea-pig can be demonstrated by intracutaneous injection, as evidenced by the development of edema and a small area of necrosis. Roemer[1] utilized this fact for a method of standardization. It is relatively accurate in experienced hands and has an advantage of economy of pigs, as several tests can be done with one pig. It is especially applicable in testing for small amounts of antitoxin. The starting-point is to determine the necrosis dose of toxin when mixed with a definite small fraction of a unit of antitoxin. A practical application of the intracutaneous method is the Schick test for the determination of immunity in man.

Standardization of Tetanus Antitoxin.—In the case of tetanus it was found that precipitated and dried toxin could be kept constant, and for testing purposes this is being sent out by central controlling laboratories such as the Hygienic Laboratory at Washington instead of the standard antitoxin. A unit may be defined as that amount of antitoxin which will protect a 350-gram guinea-pig from 1000 fatal doses of standard toxin. Note that the tetanus unit neutralizes 1000 M. L. D.'s or practically ten times the amount that a unit of diphtheria antitoxin does.

Standardization of Scarlet Fever Antitoxin.—As the scarlet fever toxin is not toxic for small animals, we cannot test the potency of the antitoxin by mixing it with a certain amount of toxin and injecting the mixture into small animals. The mixtures are tested by means of intracutaneous injections in the human skin.

The unit of a scarlatinal streptococcus antitoxin[2] is ten times the smallest amount which will neutralize 5 skin-test doses of toxin to the extent that no reaction as great as 1 cm. shall appear within forty-eight hours. Observations at twenty-four and forty-eight hours should be made. This is determined by intracutaneous injections in a series of toxin-susceptible children. A standard antitoxin should be available for comparison. Confusing reactions may result from skin-sensitiveness to horse serum and this must be controlled.

There are also obscure differences in the amounts of the different group antitoxins in persons to match the group toxins produced by a streptococcus strain. For this reason Kirkbride and Wheeler use a special breed of goats (intracutaneous test) for titration.

The Flocculation Method of Standardization.[3]—Flocculation as a result of mixing toxin and antitoxin was observed by Danysz for ricin and antiricin, by Calmette for venoms and antivenins and by Nicolle, Cesari and Debrains for other toxins and antitoxins. Ramon focussed attention on this phenomenon when he introduced simple procedures

[1] See Zingher: Jour. Inf. Dis., 1916, **19**, 557.

[2] Dyer: Pub. Health Repts., 1928, **43**, 1659.

[3] See Summary, Bayne-Jones: Newer Knowledge of Bacteriology and Immunology.

for testing diphtheria toxin and its antitoxin. The practical application of the phenomenon of flocculation depends on the fact that flocculation occurs quickest at the neutrality point. The titration of diphtheria toxin and antitoxin may be taken as an example. The first requirement is a strong toxin, 5 L+ doses or more per cubic centimeter. To titrate toxin select a quickly reacting antitoxic serum of known strength, for example a strength of 350 units per cubic centimeter. Add 2 c.c. of the toxin to each of a series of test-tubes, into which graded amounts of the antitoxin have been measured. For a toxin of moderate strength, 0.032, 0.03, 0.028, 0.026 and so forth to 0.01 c.c. of antitoxin should be added to the respective tubes. The mixtures are incubated in the water-bath at 50° C. and observed until a reaction occurs. If, for example, the first reaction occurs in the tube containing 0.03 c.c. of antitoxin the calculation is as follows: 350 (units) $\times$ 0.03 = 10.50 L+ doses per 2 c.c. of toxin or 5.25 L+ doses per 1 c.c. For the titration of antitoxin the procedure is similar: Making the same series of mixtures as above, the toxin strength being known, 5.25 L+ doses per cubic centimeter, the calculation would be 10.50 (2 c.c. in test) $\times$ 0.03 = 350 units per cubic centimeter. The method gives the binding values of toxin and antitoxin.

This test works best with horse serum, the B. fraction of concentrated antitoxin works slowly, the A. fraction not at all. The method may be applied to "toxoids" to determine the total binding value of the detoxified toxins.

The method has been mainly applied to the titration of diphtheria toxin and antitoxin. It may be applied to others. Povitsky, who has carried out these tests with remarkably clean-cut results in our laboratory, has had considerable success with scarlatinal toxin and antitoxin; others have not. Considerable investigation has been carried out as to the mechanism of the flocculation. Ramon and others believe it to be wholly due to the combination of toxin and antitoxin. Bronfenbrenner and Reichert believe the precipitate is due to bacterial antigen and antibody which parallels toxin and antitoxin values.

Use of Toxin Neutralization in the Identification of Bacteria.—As we shall see, immune reactions are commonly employed in the final identification of suspected pathogenic organisms. The determination of the morphological and cultural reactions do not suffice, because for every type of pathogenic bacterium there are similar non-pathogenic types which may often be encountered in the same area or in the same excretions which are to be examined for the presence of a pathogenic variety.

If we isolate a bacterium which has the morphological and cultural reactions of an exotoxin-producing variety, for example a suspected diphtheria bacillus, we can prove it to be a diphtheria bacillus by demonstrating that it produces a toxin which is neutralized in a susceptible animal by known diphtheria antitoxin.

Natural and Active Antitoxic Immunity.—Antitoxic immunity may be produced by the injection of sublethal doses of toxin as well as by the injection of neutral or partially toxic mixtures of toxin and antitoxin.

The latter method is utilized successfully for the immunization of man where an antitoxic immunity is absent. (See Chapter on Diptheria.)

The ease of production of antitoxin varies with different species. When natural antitoxin immunity occurs among certain individuals in a species these produce antitoxin more readily than the others. Natural immunity is never encountered among guinea-pigs and they produce antitoxin with difficulty. An antitoxin immunity does not develop until six to eight weeks. The horse, on the other hand, commonly shows a natural antitoxic immunity, and as a species produces antitoxin within a few days. If, however, an individual horse possesses no initial antitoxin he will not produce antitoxin for a few weeks. Man shows the characteristics of both the above species. Individuals possessing no antitoxin produce antitoxin in appreciable amounts only after the lapse of four to twelve weeks, whereas individuals possessing traces of antitoxin respond promptly and freely in the production of more antitoxin. This has great practical importance in the active immunization of children against diphtheria.

Passive Antitoxic Immunity.—This can be conferred by the injection of preformed antitoxin. Antitoxin can be employed as a prophylactic measure for the prevention of diphtheria, tetanus and scarlet fever; it may also be used as a therapeutic measure after the disease has developed. It must be clearly understood, however, that the therapeutic use of the antitoxin is really as a preventive measure, that is, it protects the cells from further toxic action and is of no value in restoring the cells already injured. Therefore, one can see the necessity of the earliest possible application of the antitoxin.

The amount of toxin in any case of diphtheria, for example, is comparatively small. One hundred units of antitoxin, which would neutralize fifty times the amount of toxin sufficient to kill a six-year-old child, would surely make harmless all the toxin present in the most malignant cases if it could gain access to it in time. If we gave antitoxin, therefore, as many suppose, simply in sufficient amount to neutralize the poison in the body of an infected person, comparatively small amounts would be injected, but we have to give very much more than this because of the time it requires for much of the antitoxin to reach the toxin. This can be brought into direct contact with the toxin only by being absorbed into the blood and then passing through the capillary walls to the tissue fluids and cells. The greater the quantity of antitoxin that is in the blood, the greater will be the speed that an appreciable amount will pass to the tissues. The combined endeavor of the clinical observer and the laboratory worker is to find the suitable dose which will give a sufficient concentration in the blood to neutralize, as quickly as necessary, the toxin in the tissues. In the laboratory we can test the amount of antitoxin which is absorbed into the blood from any given dose and the amount which passes out to the tissues, while the clinical observer can note the changes which take place as he watches the case after antitoxin treatment.

It is naturally a matter of great importance as to how the antitoxin

is administered. When given subcutaneously, the swelling caused by its injection rapidly disappears by the absorption of the water, but the globulins and antitoxin remain behind in the tissues because of the slow absorption of proteins. By testing many patients, it has been found that it takes twenty-four hours for the major part of the antitoxin to be absorbed by the blood from the subcutaneous tissues and some twelve hours from the muscles. For its total absorption it requires two or three days. Through the use of the Schick test, it has been determined that an injection of antitoxin given intravenously passes out to the tissue fluids about ten times as rapidly as when the dose is given subcutaneously, and four times as when given intramuscularly. A unit gives most effect when given intravenously and least when given subcutaneously. If it were not for the fact that it is more

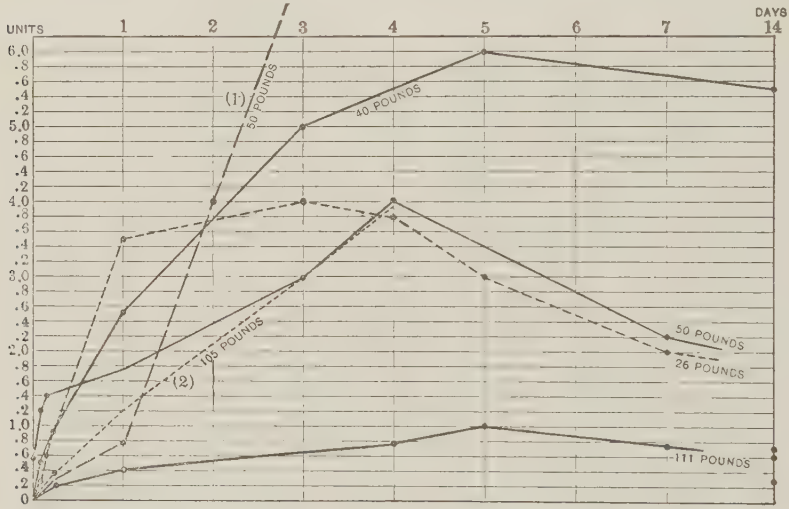


FIG. 77.—Amount of antitoxin in 1 c.c. of serum from persons, at different intervals of time (days), after a single subcutaneous injection of 10,000 units.

difficult to give it intravenously and also that sharper serum reactions occur, the intravenous method would be the only one used. Another matter which is of importance is the size of the individual treated. It is self-evident that if a child weighing twenty pounds is injected with 10,000 units, it would, on the average, have in its blood five times as much antitoxin per cubic centimeter as a person receiving the same amount who weighs 100 pounds. The influence of weight on the dose is, however, largely neutralized by the fact that diphtheria in the child is generally more dangerous than in the adult. Every minute of delay in the neutralization of the toxin in a severe case is of importance, but in a mild case, where dangerous poisoning is still remote, slight delay makes little difference. Infants and children are especially liable to laryngeal diphtheria, so that every case in a child presents a certain gravity which the adult does not present.

The last point to be considered is whether a single or a multiple dose should be given. It must be realized that antitoxin has no effect whatever on injury which has already taken place. It is as useless then as water on the ashes of a burned-out building. If the toxin is permanently united with the cell substance, antitoxin is no longer of any service. It is the early and sufficient dose which is important. When we give a divided dose, we simply get the effect of the first portion during the interval before the giving of the second dose. If the second dose had been given with the first, we would have had its effect added, and so an insufficient dose made adequate. When the first dose has been of a sufficient size, the second and third injections, though harmless, are absolutely useless. The holding back of a part of the first dose so as to give it later, simply delays its action to a time when it cannot have much, if any, effect.

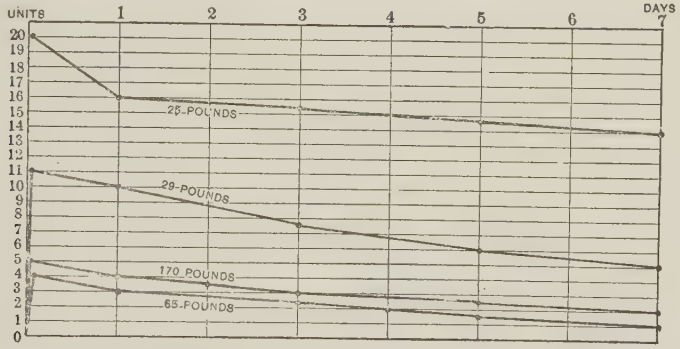


FIG. 78.—Amount of antitoxin in 1 c.c. of serum from persons, at different intervals of time, after a single *intravenous* injection of 10,000 units.

In the case of tetanus another factor is involved. If antitoxin is given intravenously only about 3 per cent. of the blood content will be found in the cerebrospinal fluid. The relative failure of tetanus antitoxin as a curative agent was probably due to this fact. The value of the intraspinous injection to obtain a local high concentration of antitoxin was definitely shown by Nicoll and Park. The antitoxin so given tends to flow to the blood until the balance is gained. It is necessary, therefore, to repeat the intraspinous injection.

Production of Antitoxin for Therapeutic Use.—A strong diphtheria or tetanus toxin should be obtained by using a culture of high toxin-producing capacity and growing it in broth under the conditions described. The following directions apply to the production of diphtheria antitoxin. The horses used should be moderately young, vigorous, of fair size and absolutely healthy. The horses are each injected with a preliminary injection of 3000 units of antitoxin homologous to the toxin to be injected. This gives a preliminary passive immunity and permits the injections of larger doses of toxin than would otherwise be allowable. The antitoxin does not interfere with further antitoxin production from subcutaneous toxin injections.

There is no way of judging which horses will produce the highest grades of antitoxin. Very roughly, those horses which are extremely sensitive and those

which react hardly at all are the poorest, but even here there are exceptions. The only way, therefore, is to bleed the horses and test their serum at the end of four weeks to two months.

Diphtheria Antitoxin.—The following scheme of injections has been used in the Health Department Laboratories with good results. The injections are given subcutaneously on the day following the injection of the antitoxin. Ten M. L. D.'s of toxin diluted with 50 c.c. of physiological salt solution are injected. Subsequent injections are given every second day, increasing the amount of toxin. The toxin is increased by 100 per cent. for the first seven injections, by 75 per cent. for the next seven injections and then by 50 per cent. for the next series. The rate of increase is then gradually lowered to 10 per cent., which is maintained until the maximum for the horse is reached. When large bleedings are begun, two injections two days apart are given and a bleeding taken seven days later. Hickson has recommended a daily injection schedule as a means of shortening the time before bleedings are begun. After the preliminary injection of antitoxin daily injections are given as indicated by the following M. L. D. values, 400, 600, 900, 1200, 2100, 3000, 4500, 6000, 7500, 10,500, 12,000, 15,000, 19,000, 24,000, 28,000, 32,000, and proportionately increases until about twenty-four injections are given. If the horse is high enough bleedings are started, and two to three daily injections are given between bleedings.

If only high grade antitoxin is wanted all horses giving less than 300 units per c.c. are discarded. A moderate percentage of horses will give 800 units or better. When the maximum is reached this may be maintained for a few months but eventually the unit content falls gradually in spite of continued injections of increasing amounts of toxin.

Tetanus Antitoxin.—This is produced exactly as in the case of diphtheria antitoxin except that one only increases each dose by 50 per cent. Good horses yield a serum containing 200 to 600 units per cubic centimeter. They continue to produce serum of great potency for long periods. The toxin has a less deleterious effect on horses than the diphtheria toxin.

Streptococcus Antitoxin (Scarlatina and Erysipelas).—Dochez injects small amounts of melted nutrient agar subcutaneously and then injects living streptococci into the agar. With repeated injections the horse develops a high concentration of antitoxin and some antibacterial antibodies. Ramon recommended the use of tapioca with diphtheria toxin and this procedure has been used in streptococcus antitoxin production.

Horses may be injected simply with increasing doses of streptococcus toxin as is done in diphtheria antitoxin production.

Moser's antistreptococcus serum was a combined antitoxic and antibacterial serum. We are producing a similar serum by alternate injections, killed streptococci intravenously and toxin subcutaneously.

The Handling of the Antitoxic Serum or Plasma.—For the collection and preservation of the antitoxin the blood is withdrawn from the jugular vein by means of a sharp-pointed cannula, which is plunged through the vein wall, a slit having been made in the skin. When serum is to be obtained, the blood is carried by a sterile rubber tube into large Erlenmeyer flasks, held slanted, or into cylindrical jars, and allowed to clot. The serum is drawn off after four days by means of sterile glass and rubber tubing, and is stored in large flasks. When the globulins are to be separated the blood may be added directly to one-tenth of its volume of a 10 per cent. solution of sodium citrate. This prevents clotting of the blood. The heavy cells settle to the bottom and the clear plasma is withdrawn. Tricresol 0.3 per cent. is added as preservative. Serum can be utilized immediately after testing for unit content and sterility.

Separation and Concentration of Antitoxin.—Ordinary antitoxic serum contains serum globulins, albumins, nucleoproteins, cholesterin, lecithin, traces of bile pigments and salts, non-protein compounds, inorganic salts and water. Pick and Atkinson showed that the antitoxins were present in the globulin fraction and that during immunization the content in globulins increased.

Gibson and Banzhaf then showed that the fraction of globulins insoluble in saturated salt solution carried no antitoxin: that is, the pseudoglobulins not the euglobulins carried the antitoxin. The original concentration method of Gibson and Banzhaf was therefore to precipitate out the globulins by 50 per cent. concentration of ammonoium sulphate, dissolve out pseudoglobulins with saturated salt solution and dialyze to remove the salt. Banzhaf then showed that heating to 57° to 62° C. for one hour rendered some of the pseudoglobulins fraction insoluble in sodium chloride, thus he attained a greater purification. The methods of Atkinson and Gibson and Banzhaf were worked out in the Laboratories of the Health Department of New York City and Banzhaf's continued experiments have resulted in the adoption of the following procedure now generally in use.

To the citrated plasma, containing 0.3 per cent. tricresol, add an equal volume of water. To 7 parts of this mixture add 3 parts of a saturated solution of ammonium sulphate (30 per cent. saturation). Heat at 57° to 62° C. for one hour, filter while hot. This gives two fractions, thus:

A. Precipitate.	B. Filtrate.
(Contains euglobulins, some pseudoglobulins and fibrinogen).	(Contains pseudoglobulins and albumins).
The precipitate is washed with 30 per cent. saturated ammonium sulphate.	Add washings from fraction A.
Dissolve in water and add NaCl to saturation (dissolves out the pseudoglobulins). Filter.	Add sufficient saturated ammonium sulphate solution to raise concentration to 50 per cent. (precipitates the pseudoglobulins). Filter.
Add acetic acid (to precipitate pseudoglobulins). Filter.	Precipitate pressed between pads to remove excess ammonium sulphate solution.
Precipitate pressed to remove excess water and acid.	Place in dialyzing bag, dialyze to remove salts.
Place precipitate in dialyzing bag and dialyze to remove salts.	Add NaCl to 0.8 per cent. and preservative, tricresol 0.4 per cent.
Add sodium chloride 0.8 per cent. and preservative tricresol 0.4 per cent.	

Filter fractions separately through paper pulp and Berkefeld filter (latter to remove bacteria). Test fractions for unitage and sterility.

Both fractions may be mixed together. In practice, because the "A" fraction has a lower unitage per gram protein and also because it is finished later, the two are not mixed. The A fractions from successive preparations are usually accumulated and then mixed.

Advantages of Concentration of Antitoxin.—With the method just given the finished antitoxin has 4 to 6 times the unit content of the original plasma. The total proteins as compared with serum antitoxin having the same total units is reduced about one-half. The result is that a larger unit dose may be given with the introduction of less foreign protein. This has resulted in a considerable decrease in the serum reactions, especially rashes. The heating also reduces the capacity of the proteins remaining to produce rashes and fever after injection.

Toxins Other than those of Microbal Origin and their Antitoxins.—Plants as well as animals secrete substances which have many or all of the characteristics of microbal exotoxins. They may be characterized as phytotoxin (plant origin) and zoötoxins (animal origin). Examples

of the former are ricin from the seed of the castor oil plant (ricinus communis), crotin, from the seed of a tree (croton tiglii) and abrin from the seed of the Indian licorice (abrus precatorius). They are toxic and when injected, stimulate the production of an antitoxin. As they agglutinate or hemolyze red cells, and because they are relatively stable, they have been utilized to study the laws of combination of toxin and antitoxin, by means of test-tube reactions. The zoötoxins are the venoms of snakes, spider, scorpion, etc. The snake venoms contain toxic elements active against nerve cells, red cells and endothelium. They differ from microbal exotoxins in being relatively heat resistant, withstanding heating up to 75° C.; cobra venom is not destroyed even by heating to 100° C. for a very short time. Antitoxins of moderate potency can be produced for each of these venoms. As has been noted there is some similarity between the action of toxins and of enzymes. Antienzymes can be produced by animal injections which are specific and will neutralize the enzyme injected.

CHAPTER IX.

ANTIMICROBAL OR ANTIPROTEIN SUBSTANCES.

AGGLUTININS.

By the phenomenon of agglutination is meant the aggregation into clumps of microörganisms uniformly dispersed in a fluid. If the organisms are motile they become immobile during this process. Many substances other than those in serum cause the agglutination of cells. We as bacteriologists are interested in these chiefly because they may cause us to believe we have noted agglutination phenomena due to serum antibodies when really they were due to non-specific chemical substances.

This phenomenon (agglutination), while it had been noted by earlier observers (Charrin and Roger in 1889), was first extensively studied by Gruber and Durham in 1896, who determined that the serum of those passing through certain infections contained a specific substance (agglutinin) which caused the infecting organisms to clump. Several months later Widal reported that in typhoid fever the demonstration of the development of agglutinins could be used for diagnostic purposes. It was thus demonstrated by these studies and those of Grunbaum, Bordet and others that through agglutinins a new means was available for the identification of bacteria and in many cases the nature of the infectious organism causing disease.

Agglutinogen.—The antigenic substance stimulating the production of agglutinins is called the agglutinogen. It is found in the living or dead microbal cell and appears to be released in cultures only on the dissolution of the organism. Heat and chemicals tend to destroy or alter agglutinogenic action. According to the Ehrlich hypothesis we may look upon the agglutinogen as a molecule having a haptophore group through which it combines with the receptor of the body cell. Evidence has been advanced that the specific agglutinogen is contained in the ectoplasmic portion of microörganisms.

Nature and Structure of Agglutinins.—Agglutinins resist heating to 60° C., but above this temperature they are gradually destroyed, the destruction being complete at 75° C. They are not dialyzable and are precipitated with the globulin fraction of the serum. They are most active in a neutral or slightly acid menstruum. Alkalis tend to inhibit their action. They are the products of cellular activity and according to Ehrlich are more complex than the antitoxins in that they possess both a haptophore group by which they combine with the receptor of the microbal cell, and also a ferment portion or zymophore group to which they owe their action. Ehrlich therefore termed the agglutinins "anti-

bodies of the second order." As under certain circumstances, such as the application of heat, acids, etc., agglutinins apparently lose their clumping but retain their combining power, Ehrlich drew a parallel with toxin and toxoids and called these altered agglutinins, "agglutinoids," that is, the zymophore or active portion might be destroyed but the haptophore fraction retain its affinity. This conception of agglutinins, like that of toxins cannot be sustained in the light of later work (see below). Agglutinins are specific within certain limitations and the combination with agglutinogen is approximately quantitative. They deteriorate more rapidly in dilute solutions, less rapidly in the undiluted serum. If the serum is dried and stored *in vacuo* the agglutinins will persist for long periods of time.

Mechanism of the Agglutination Reaction.—Agglutination is not a vital phenomenon. The microörganisms play a purely passive role.

The observations of Bordet have shown that the earlier idea that agglutination is directly due to the agglutinins to be untenable. He showed very definitely that agglutination was a physical phenomenon and that the combination of antigen and antibody was one phase, and agglutination another. Thus if a suspension of bacteria and a sample of serum be dialyzed until all salts are removed their mixture will not result in agglutination, unless salt be added. Or if the dialyzed mixture be centrifuged to remove the bacteria, and the sedimented bacteria suspended in salt solution agglutination will promptly occur. If sufficient bacteria have been used the supernatant fluid is free of agglutinins as shown by the absence of agglutination when salt and fresh bacteria are added. In other words combination occurs in absence of salts but agglutination only in their presence or, otherwise stated, agglutination is a clumping of the bacterium-antibody complex by salt action. These observations led to a comparison of the agglutination phenomenon to the flocculation of one colloid by another and the flocculation of colloids by salts and acids (electrolytes).

An explanation of the mechanism of agglutination must deal with:

1. The factors which are responsible for the stability of a suspension of normal bacteria.

2. Any changes in these factors due to the agglutinins in immune serum that may destroy the stability.

The stability of a suspension of normal bacteria depends upon the equilibrium between forces of repulsion and forces of attraction. Bechhold showed that bacteria, whether heated or unheated, are electronegative. Particles carrying a similar electric charge repel each other, and this is the repulsion force. The neutralization of electric charges, or the reduction of potential, was found to be insufficient by itself to produce agglutination (Neisser and Friedmann, Buxton, Northrop and De Kruif), and another force, one of attraction, or the "cohesive force" of Northrop and De Kruif, had to be assumed. The cohesive force (probably surface tension or surface energy) can be measured. Thus, the factors influencing the equilibrium in favor of greater repulsion or lessened cohesion lead to flocculation.

By measuring the potential as well as the cohesive force, Northrop and De Kruif[1] were able to determine a great many of the factors involved in the maintenance of stability as well as the production of flocculation in a suspension of bacteria.

Potential is measured by the rate of migration in an electric field.

Cohesive force is determined by measuring the force required to tear apart two glass plates covered with a film of the bacteria and immersed in the solution under investigation.

When agglutinins are added the globulin carrying the agglutinin unites and reacts on the periphery of the microörganism. This gives a new complex. It has been shown that the addition of salt (electrolyte) in the amount employed in "normal" saline depresses the cohesive force in an unsensitized suspension of bacteria to such an extent, that regardless of any reduction in potential agglutination does not occur. However, the sensitized bacteria (bacteria and agglutinins) are protected from the depressing effect of this concentration of salt, so that when the potential is reduced to the critical zone agglutination occurs, because the cohesive force is now relatively the stronger. Jones, in studying the phenomenon of flocculation, showed that collodion particles covered with egg-albumen are specifically agglutinated with antiegg-albumen serum. Bacteria treated in a similar manner take on the immunological specificity of the protein covering. These observations indicate that when specific immune body carrying globulin combines with the substance of the bacterium, the bacterium, in a sense, may be considered denatured, a term suggested by some observers, but in reality there is a new complex.

The above outlines the mechanism of serum agglutination. There must be some chemical phenomenon at the basis of agglutination, which would explain the specific combination of antigen and antibody.[2]

Agglutination of bacteria can be caused by non-specific means which influence the balance of the two factors discussed above. The acid agglutination (Michaelis) is the agglutination of the bacterium at the iso-electric point of the colloidal constituents.

Agglutination in the Body.—Several investigators have definitely shown that agglutination does occur *in vivo*. Bull has studied the subject in detail. He found that when typhoid bacilli are injected into normal rabbits they are agglutinated rapidly in the circulating blood and accumulate in the capillaries of various organs where they are taken up by phagocytes. In immunized animals the phenomenon is more marked. If antipneumococcus serum is injected into rabbits having a pneumococcus septicemia a similar phenomenon results. Similar results have been obtained with other bacterial types. It would seem, therefore, that agglutinins have a function in the body; to agglomerate the bacteria and thus aid in their removal by phagocytic cells.

[1] Northrup and De Kruiff: Jour. Gen. Phys., 1922, **3**, 5, 127. Shibley: Jour. Exp. Med., 1926, **44**, 667. Jones: Jour. Exp. Med., 1928, **48**, 183.

[2] Phillip, J. C.: Physical Chemistry, p. 218; see also Bacterial Constituents below.

Measurement of Agglutinins or Titration of an Agglutinating Serum.—The agglutinating power of a serum is measured by determining the highest dilutions in which it still exhibits this quality. The titer of the serum as commonly used implies the highest dilutions in which complete agglutination, or some arbitrarily selected degree of agglutination, occurs or, in another way, the titer is the smallest amount of serum which will agglutinate an arbitrarily selected standard amount of culture. The titer will vary according to the three factors, density of suspension, temperature of incubation and time of incubation. The denser the suspension the more agglutinins will be required. Agglutination proceeds relatively more rapidly as the temperature is raised from 20° to 55° C. Within limits, time of incubation likewise influences the degree of reaction. The reaction may be observed under the microscope, "microscopic method," or by the eye using test-tubes or slide "macroscopic methods" (see below, Technic of Agglutination Reactions).

Pre-zone or Proagglutinoid Phenomenon.—Not infrequently it is observed that low dilutions of serum cause poor or even no agglutination, whereas higher dilutions will cause better or even a complete reaction. This phenomenon is explained by Ehrlich as due to the presence of agglutinoids which, having a greater affinity for the bacteria, combine and prevent the action of the agglutinins or may also be attributed to presence of a protective colloid. With either of these hypotheses we should assume that the substances are present in insufficient amounts in the higher dilutions. Similar zones are noted in the flocculation of one colloid by another, when one or the other is in excess. An excess of agglutinins alone is not sufficient explanation, however, as the occurrence of pre-zone phenomena does not parallel the titre of different sera.

Specific (Major), Group (Minor or Common) and "Normal," Agglutinins.—Agglutinins may exist in the blood of individuals who so far as we know have not been infected by (carrier state or disease) or injected with any of the bacteria acted upon by these agglutinins. These agglutinins are termed "normal agglutinins." Thus the serum of man may agglutinate B. typhosus, B. coli, etc. in dilution of 1 to 2 or 1 to 5. The serum of a horse may agglutinate such bacteria in dilutions of 1 to 100 or even 1 to 1000 or more. The possession of such agglutinins has been attributed to the stimulation resulting from the continued absorption of bacteria of the colon group from the intestinal tract. Such an hypothesis, however, does not fully explain why most normal horse serums agglutinate glanders bacilli in such high dilutions as 1 to 500 or more.

The agglutinins which develop after infection or after inoculation are commonly designated immune agglutinins. The immune agglutinins which result from the stimulus resulting from infection or injection of a bacterium act not only on this bacterium but also on related bacteria. This gave rise to the conception that some of the agglutinins act only on the specific component of the stimulating bacterium, specific or major agglutinins, and that some act on the substance or substances common to this bacterium and related varieties, group, common or

minor agglutinins. Due to the influence of Ehrlich's hypothesis this was attributed to the agglutinogenic action of individual components of the bacterium. This conception is schematically represented in Fig. 79. How far this conception is justified will be evident from the discussion (below) on bacterial constituents.

As a rule the degree of group agglutination is less marked than is the case with the specific variety. Successive dilutions will serve in such instances to allow of a differentiation between specific and group action. Not infrequently, however, cross agglutination with a related variety may be so high that one must resort to agglutinin absorption for differentiation.

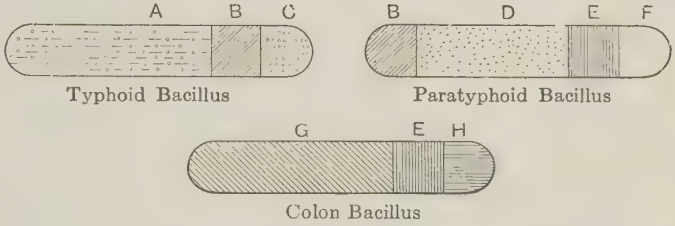


FIG. 79.—Specific and common agglutinogens diagrammatically shown.

Several factors influence the degree of group action of agglutinating antiserums. The duration of agglutinogenic stimulation, that is the duration of infection or the number of injections, is likely to have a marked influence. A graphic illustration of this is given in Fig. 80. Another factor is the host species. Thus the rabbit as a rule produces

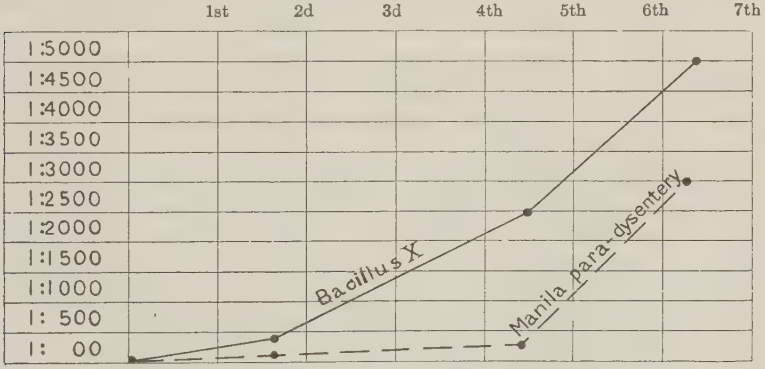


FIG. 80.—A young goat was used for the injection of the colon bacillus X. The great accumulation of common agglutinins for the paradysentery bacillus in the fifth week of the injections of the bacillus X is very striking.

• Tests made.

less group agglutinins than the goat and the goat less than the horse. There is seemingly a relationship between the tendency to group agglutinin production and the relative content in normal agglutinins. Thus as a rule the rabbit possesses the least normal agglutinins, the goat more and the horse most.

A horse after injection with a B. paradysenteriæ culture may yield a serum giving results as follows:

AGGLUTININS IN THE SERUM OF A HORSE INJECTED WITH PARADYSENTERY BACILLUS, CULTURE TYPE MANILA.

Culture.	After 18 injections.			After 21 injections.		
	1:3000	1:5000	1:10,000	1:3000	1:5000	1:10,000
Paradysentery type Manila .	++	−	−	++	++	++
Colon bacillus, type X . .	++	++	−	++	++	++

Careful titration might have shown that such a horse had an excess of agglutinins for B. coli before immunization in which case the high "group" reaction is really the summation of group and normal agglutinins.

The above discussion assumes that the cultures employed are nearly equally agglutinable. If the heterologous organism is very easily agglutinable, one may even obtain the apparent paradox that an antiserum agglutinates it better than it does the serum culture.

Bacterial Constituents in Relation to Specificity.—The question of the specificity of antibacterial antibodies is related to the constitution of the bacterial cell. This subject may be outlined here, as the knowledge we possess applies to all antibacterial antibody phenomena.

The bacterial cell contains lipoidal, protein or protein-like, and carbohydrate materials. The evidence available does not indicate that pure lipoids are antigenic. They may, however, play a rôle in the phenomenon of complement-fixation.[1] The lipoids in combination with protein may markedly alter the antigenic character of a protein. The protein or protein-like materials of bacteria commonly spoken of as bacterial nucleoprotein is probably a mixture of different substances and constitutes the bulk of the bacterial cell. Some at least of this substance is antigenic.

This substance as a whole is low in stimulating capacity, as shown by the relatively long time needed to stimulate appreciable amounts of antibodies, but some separated bacterial proteins may be highly antigenic. The carbohydrate constituents, though known since the early days of bacteriology, only recently have been recognized generally as playing a specific rôle in immune reactions. Pick, in 1912, obtained a substance from typhoid bacilli which was not affected by pepsin, by trypsin or by boiling and which gave none of the protein reactions. This substance would not stimulate the production of antibodies, but would precipitate specifically with antityphoid serum. Zinsser obtained similar results with what he termed "residue antigens." Avery and Heidelber-

[1] The term antigen from its etymology should be applied only to substances which have the ability to stimulate the production of antibodies. Usage has broadened the term to include substances which do not possess this ability but which give specific reactions with antibodies. This divergence from the original meaning of the term is particularly noticeable in the case of the Wassermann test and other similar diagnostic procedures. To be correct, the term specific reagent should be applied to such so-called antigens as well as to the soluble specific substances described in this section.

ger have added greatly to the chemical knowledge of this substance in relation to the specificity of immune reactions and their work has given a definite basis for generalization. They separated a polysaccharide gum which in dilutions of the dried substance 1 to 1,000,000 or less gave specific precipitation or complement-fixation reactions, that is, bound antibacterial antibody specifically, although it had no demonstrable antigenic capacity. The immunological constituents of many bacteria have been studied, pneumococcus, Friedländer bacillus, streptococci, staphylococci, typhoid bacillus, influenza bacillus, etc. As so many varieties have been studied, it would seem justifiable to generalize the results and assume that the facts obtained are applicable to all bacteria.

The antigenic action of bacterial substance is due to a protein-carbohydrate complex. If this complex be dissociated the protein will be found to be antigenic but the carbohydrate will not, though it will bind antibody.

The antibody, stimulated by the unchanged protein-carbohydrate complex, is, as a rule, sharply type specific; for instance, the antibody stimulated by the unaltered complex in pneumococcus Type I will only react with pneumococcus Type I not with other pneumococci. The antibodies stimulated by this complex will be bound and will react with the unaltered bacterial cell and will be bound and react with the specific carbohydrate ("soluble-specific substance," Avery and Heidelberger), though, as stated above, this substance will not stimulate antibody production. The protein and carbohydrate combination is relatively unstable, and if separated cannot be recombined. If the separated constituents are injected together only antiprotein antibodies (usually common or group) are stimulated. The antibodies stimulated by the separated protein[1] constituents are largely group or common in character, reacting with similar varieties or extracts of their protein constituents.

Thus the antiprotein antibodies stimulated by the protein of pneumococcus Type II will react with all pneumococci or their protein substance. These antibodies, however, will not combine with the carbohydrate substance and, therefore, will give no reactions with this substance.

In infection or in the process of artificial immunization both specific and group antibodies are stimulated. In the former case lysis and separation of the protein-carbohydrate complex must be occurring in the body In the latter the same phenomenon takes place, but the bacterial suspension may contain the separate elements, as well, due to autoferment action (autolysis). Evidently the ideal antigen for the stimulation of specific antibacterial antibodies is the unaltered complex which, for practical purposes, means the whole bacterial cell freed from all associated products of lysis.

Specificity is apparently a function of the ectoplasm of the microbes. Considerable of the published work has been done with bacteria which

[1] For details and limits of this general statement consult references given.

under the proper conditions have capsules easily demonstrable by staining methods, and it might seem implied that the absence of a demonstrable capsule by the ordinary staining methods means a loss of type specificity and, therefore, of soluble specific substance. This is not the case, as a pneumococcus growing on ordinary culture media and without such demonstrable capsule may possess specific antigenic capacity. However, it is possible to degrade a pneumococcus, so that practically all type specificity is lost, and it may be presumed that this is due to the complete loss of ectoplastic (capsular) substance. With motile bacteria, degradation or loss of specific antigenic capacity is associated with the loss of flagella (loss of motility). It may be presumed, therefore, that flagella contain specific substance, though this has not been demonstrated by actual separation of such substances. All the specific substance may not be in the flagella, as the loss of motility and associated loss of specificity may be further associated with loss of other ectoplastic material.

In the above summary it was noted that, in general, specificity is a function of the carbohydrate constituent, group or common reaction, a function of the protein constituents. There has been observed one definite instance where group relationship is due to likeness of carbohydrate constituents. Avery found that the pneumococcus Type II and B. pneumoniæ (Friedländer) group B contained a chemically very similar carbohydrate fraction which gave marked cross reactions. It would seem that other instances of very close relationships among bacteria are not wholly explainable by the possession of common protein substances.[1]

Absorption or Adsorption of Agglutinins.—Early observers had noted that agglutination was followed by reduction or disappearance of agglutinins from the menstruum in which the reaction took place. Castellani studied this phenomenon and showed that absorption could be applied as a means to differentiate with accuracy between specific and group agglutination. The specific agglutinins and also the common agglutinins

[1] Cholera Vibrio. Landsteiner and Levine: Proc. Soc. Exp. Biol., 1926, **24**, 248. . . . Meningococcus. Zinsser and Parker: Jour. Exp. Med., 1923, **37**, 275. Przesmycki: Jour. Inf. Dis., 1924, **35**, 537. . . . Staphylococcus. Zinsser and Parker: Jour. Exp. Med., 1923, **37**, 275. . . . B. influenza. Zinsser and Parker: Jour. Exp. Med., 1923, **37**, 275. . . . Smegma bacillus. Freund: Proc. Soc. Exp. Biol., 1926, **24**, 249. . . . Tubercle bacillus. Laidlaw and Dudley: Brit. Jour. Exp. Path., 1925, **6**, 197. Zinsser: Jour. Exp. Med., 1921, **34**, 495. . . . B. proteus. Przesmycki: Compt. rend. Soc. de biol., 1926, **95**, 744. Furth and Landsteiner: Jour. Exp. Med., 1928, **47**, 171. . . . B. typhosus and paratyphosus. Landsteiner and Furth: Proc. Exp. Biol., 1928, **25**, 565. Nelson: Jour. Exp. Med., 1928, **48**, 811. Zinsser and Parker: Jour. Exp. Med., 1923, **37**, 275. Furth and Landsteiner, 1928, **47**, 171. Pneumococcus. Heidelberger and Avery: Jour. Exp. Med., 1923, **38**, 73; 1924, **40**, 301. Zinsser and Parker: Jour. Exp. Med., 1923, **37**, 275. . . . Streptococcus. Zinsser and Tamiya: Jour. Exp. Med., 1925, **42**, 311. Lancefield: Jour. Exp. Med., 1925, **42**, 377; 1928, **47**, 91. Hitchcock: Jour. Exp. Med., 1924, **40**, 445 and 575. . . . Friedländer bacillus. Heidelberger, Goebel and Avery: Jour. Exp. Med., 1925, **42**, 701 and 727. Mueller, Smith and Litarzek: Proc. Soc. Exp. Biol., 1924–1925, **22**, 373. . . . Yeasts. Mueller and Tomcsik: Jour. Exp. Med., 1924, **40**, 343. . . . Somatic and flagellar agglutinin. Smith and Reagh: Jour. Med. Res., 1903-1904, **10**, 89. Nelson: Jour. Exp. Med., 1928, **48**, 811. Orcutt: Jour. Exp. Med., 1924, **40**, 43.

which are stimulated by the infecting or injected bacterium should be bound by the homologous organism; in other words, if we add to a serum a large amount of its specific organism and then incubate we shall find that the clear serum obtained by centrifuge or filtration will no longer agglutinate either the specific organism or any of the related types previously agglutinated through the action of the group agglutinins. If we absorb similarly the serum with a related type we find the specific agglutinins will not be appreciably reduced, whereas the group agglutinins for the absorbing strain will have partly or wholly disappeared. Examples with moderate and extreme group agglutination are shown in the following tables.

ABSORPTION OF AGGLUTINATING SERUM OBTAINED BY INJECTION OF B. TYPHOSUS.

	Highest dilution giving complete agglutination before absorption.	Absorbed by B. typhosus.	Absorbed by B. coli.
B. typhosus . . .	1 to 5000++	1 to 50−	1 to 4000++
B. coli	1 to 1000++	1 to 50−	1 to 50−

ABSORPTION OF AGGLUTINATING SERUM OBTAINED BY INJECTION OF B. PARATYPHOSUS B.

	Agglutination before absorption.	Absorbed by B. paratyphosus "B."	Absorbed by B. pestis caviæ.
B. paratyphosus B.	1 to 10,000++	1 to 50−	1 to 8000++
B. pestis caviæ	1 to 10,000++	1 to 50−	1 to 50−

We have stated that the absorption by a heterologous bacterium will result in the absorption of the common or group agglutinins for itself. The absorbing type may absorb the common agglutinins for one or more other related types as well.

One may obtain at times the following apparently contradictory absorption results.

Horse Serum.—Horse immunized with B. typhosus.

	Agglutination before absorption.	Absorbed by B. typhosus.	Absorbed by B. coli (m)
B. typhosus . .	1 to 10,000++	1 to 50−	1 to 8000++
B. coli (n) . . .	1 to 1,000++	1 to 50−	1 to 50−
B. coli (m) . . .	1 to 1,500++	1 to 300+	1 to 50−

Why did B. typhosus not remove all group agglutinins for B. coli (m). The explanation is that the agglutinins remaining are normal agglutinins for which the homologous type has no absorbing content. Their normal presence was due to the stimulation by bacterial substance unrelated to that contained in the typhoid bacillus.

This is shown by testing the serum before immunization was started thus:

	Agglutination before absorption.	Absorbed by B. typhosus.	Absorbed by B. coli (m).
B. typhosus	1 to 500++	1 to 25−	1 to 25−
B. coli (n)	1 to 600++	1 to 25−	1 to 25−
B. coli (m)	1 to 500++	1 to 250++	1 to 25−

Such unrelated normal agglutinins may increase during immunization because of a little understood non-specific stimulating effect of foreign protein. The technic of agglutinin absorption, its use as a practical test and the deductions which may be drawn from such tests are given later.

Variations or Modification of Agglutinability. — As a rule bacteria are relatively resistant to agglutination when freshly obtained from the body, especially if isolated from the blood or tissues. When cultivated on artificial media they become more sensitive to agglutination, and after a shorter or longer period many varieties tend to agglutinate spontaneously when grown in broth or suspended in salt solution. At times, organisms from the blood stream may be practically inagglutinable, becoming more agglutinable after cultivation for some time. The agglutinability of a culture can be varied; the conditions, as a rule, which tend to raise its virulence also make it less agglutinable (growth on blood immune serum, animal passage, etc.). As we shall see, these same means make a bacterium less susceptible to the action of other antibacterial antibodies. This suggests that the process is one of adaptation and possibly the production of anti-substances which neutralize or prevent the combination of the antibodies of the host. (See below.) It does not follow that because bacteria are less agglutinable that they are less antigenic. The presence of a capsule may interfere with or prevent agglutination. The capsule, developing best in body fluids or tissues, is probably a protective substance. Porges has outlined a method for the removal of the capsule as a preliminary to agglutination.

An interesting modification of the resistance of bacteria to agglutination and other antibacterial antibodies is seen when bacteria are cultivated in immune sera. If grown in broth containing a sufficient concentration of the serum a culture may not only become highly resistant to agglutination but may fail almost completely to absorb agglutinins. The agglutinability and capacity to absorb agglutinins eventually return after cultivation on ordinary media.

Dead bacteria agglutinate well, although somewhat more slowly, if killing is done by the addition of 0.25 per cent. carbolic acid or preferably 0.1 per cent. formalin. If killed by heat the temperature should not exceed 55° C. Heating to 60° C. diminishes their agglutinability.

Dreyer found that if a twenty-four-hour bouillon culture of B. coli required 1 part of agglutinin to agglutinate it, then if heated to 60° C. it required 2.3 parts; if to 80° C., 18 parts; if to 100° C., 24.6 parts. He found the surprising fact that long heating of the culture restored to some extent its ability to be agglutinated by smaller amounts of agglutinins. Heated thirteen hours to 100° C. the culture was agglutinated by four parts.

The Development of Agglutinins.—Appreciable agglutinins do not develop until toward the end of the first week of infection, and the agglutinin content tends to rise if infection continues, reaching its acme at the height of the disease or somewhat later. Animals show the development of agglutinins in about the same length of time after immunization

is begun. Each successive injection tends to stimulate the production of further agglutinins until a considerable concentration is present. A limit is reached, however, and as a rule it is difficult to maintain the maximum for any length of time.

Different varieties of microörganisms vary in their agglutinogenic capacity. Thus the typhoid-paratyphoid group has a high agglutinogenic value, the streptococcus-pneumococcus group a relatively low power. Infection by members of the former causes a relatively high agglutinin content, whereas infections by the latter cause the production of only a relatively low agglutinin content. The same result is obtained after intensive immunization of animals, an antityphoid serum giving agglutination in dilutions of thousands or tens of thousands being easily obtained, whereas an antipneumococcus serum agglutinating above 500 to 600 is less easily produced.

The amount of infection or injected antigen bears a relative proportion to the amount of agglutinins produced. As the amount of antigen is increased there is an increase of agglutinin production, but the latter increase gradually diminishes until even a very marked increase of antigen gives little associated increase in agglutinin.

Practical Applications of the Agglutination Reaction.—The agglutination reaction controlled by agglutinin absorption is the method employed for the determination of the identity of culturally similar microörganisms. By this means we have determined, for instance, that all races of B. typhosus are alike; that is, that B. typhosus is a distinct type or entity. In this connection it is worthy of note that a group of bacteria may differ culturally, for instance, in their fermentative reactions and still be found to be identical agglutinatively. The agglutinative method likewise allows us to separate culturally similar members of a definite bacterial species into subgroups, an example being the pneumococcus, of which we have a series of distinct types, and also a group of agglutinatively dissimilar varieties.

In some instances, we must, because of the marked degree of cross or group agglutination shown by related groups, resort to absorption before differentiation into groups is possible.

It is obvious that having knowledge of the kind above outlined we can then utilize the agglutination reaction to identify a definite type such as B. typhosus or determine into which subgroup a bacterium belongs, for example, the pneumococcus. With certain groups we shall know that the subgroup cannot be determined with certainty unless we employ the absorption method (meningococcus). With other species we will be satisfied with morphological or a cultural identification because we do not possess the necessary knowledge for further agglutinative identification or because the knowledge we have indicates the existence of a multiplicity of races. Finally, we may not be able to employ agglutination for identification because a bacterial type or group (at least most strains) do not agglutinate well or because of their character (clumping and adhesiveness, etc.) a good suspension is difficult or

impossible to make (B. anthracis). In utilizing the direct method for identification the precautions are: (1) The serum employed must be of high titer to overcome the tendency to resist agglutination shown by freshly isolated strains. The serum from infected human beings is usually unsatisfactory for this purpose because the titer is much lower than that obtainable by intensive immunization of animals. (2) The range of specific and group (as well as normal) agglutinins must be known, that is, determined by tests using homologous and related types. (3) The unknown type must agglutinate considerably beyond the range of group action before identification is considered sufficient for practical purposes.

As was noted previously some closely related bacteria may show such marked cross-agglutination that separation by direct agglutination is difficult or even impossible.

The reverse application, viz., the determination of the kind of agglutinins present in a patient's serum, can be utilized for the diagnosis of disease. In this method we utilize a known bacterium to identify the agglutinins. It should be clearly understood that this method by itself does not possess final or absolute value. Thus we test a person's serum and find a considerable content of "typhoid" agglutinins. Unless we have isolated an infecting typhoid bacillus we cannot prove that they are not group agglutinins, developed because of an infection by B. paratyphosus or some other similar but infrequent type. Taking the findings, however, in connection with clinical facts and our knowledge that the majority of continued fevers of typhoidal character are (in this vicinity) due to B. typhosus the presence of agglutinins becomes the strongest possible evidence of the existence of an infection due to B. typhosus. If we suspect the infection to be due to a paratyphoid type we can determine whether B. typhosus or B. paratyphosus "A" or "B" is agglutinated in higher dilutions. The presumption is that the type agglutinated in highest dilutions is the infecting type. This is only a presumption, however, as paradoxical results may occur, as we have said, due to a high group agglutinin content or oversensitiveness of one culture. Finally, it is possible that a fourth related type may have been the infecting organism, that is the agglutinins acting on the three types mentioned may be group agglutinins. Absorption of agglutinins was advocated by Castellani as a means of differential diagnosis. This is discussed below.

The direct agglutination reaction for diagnostic purposes has only a limited application. It can only be applied in diseases in which agglutinins are freely produced. Even here its practical value may be lessened because other simple methods are applicable earlier in the disease. Before it can be applied to a specific disease we must first determine the range of agglutinins normally possessed by uninfected individuals; knowing this we designate a minimum diagnostic dilution for a positive test. Although this method may be of little value in the early diagnosis of such acute diseases as cholera, it may be of the greatest value in

making a *post facto* diagnosis. The agglutination test is chiefly used in typhoid and paratyphoid fevers. The use of the typhoid-paratyphoid vaccine creates a difficulty. The agglutination test is also of great help in undulant fever (Malta fever).

Technic.—The agglutination reaction may be observed in the hanging drop, using the microscope ("microscopic method"), or observed in the test-tube with the eye alone or aided by a low power lens ("macroscopic tube method"), or on a glass slide ("slide agglutination method"), a modified macroscopic method.

Obtaining the Serum or Blood.—Small amounts of serum may be obtained by pricking the finger or ear and collecting the blood in a Wright capsule. If the serum is not clear after clotting, the clot and red cells can be thrown down in the centrifuge. One cubic centimeter of blood can be obtained in this way. Dried blood drops, as advocated by Wyatt Johnson, are extensively employed in the Widal reaction. The finger or ear is pricked and the blood allowed to drop on thin metal strips or glass slides. Two separate drops should be collected.

Larger amounts of blood may be obtained from a vein. Collection by exsanguination is the method commonly employed with animals, the blood being obtained from the carotid or from the heart by suction. The blood is collected in a tube or bottle, slanted until firmly clotted and the tube or bottle stood upright for the serum to separate.

Dilutions.—With very small amounts of serum the capillary tube method of Wright may be employed (see under Opsonins). For large amounts the ordinary laboratory pipettes can be employed. The range and number of dilutions will depend on the specific purpose. The following suggestions may be of help to the beginner. If from the original serum we prepare a 1 to 10 dilution (1 part of serum plus 9 parts of physiological salt solution) and then prepare from this a 1 to 100 dilution, from this a 1 to 1000 dilution and from this a 1 to 10,000 dilution, etc., the intermediate dilutions are easily prepared thus:

1 to 10 dilution . . .	1 part	1 part	1 part	1 part	1 part
plus saline	1 part	2 parts	3 parts	4 parts	5 parts, etc.
gives a dilution of . .	1 to 20	1 to 30	1 to 40	1 to 50	1 to 60

Intermediate dilutions are similarly prepared in the hundreds, thousands, etc., thus, 1 part of 1 to 10,000 dilution plus one-tenth part of saline gives a 1 to 11,000 dilution, etc.

In setting up the reaction we usually add the bacteria in suspension, and (see below) this makes a further dilution. This final dilution is used for recording purposes.

Dried blood is diluted by adding water to the drop. This dissolves out the hemoglobin-tinged serum and the dilution is estimated from the color. Each examiner should educate his eye by making a few accurately measured dilutions and note the color. Thus, a 1 to 10 dilution is a distinct cherry red color. Subsequent dilutions are made by mixing a loop of this with the appropriate number of loops of saline to give the higher dilutions of serum required. The addition of an equal loop of broth

culture to the serum dilutions doubles the dilution. The dried blood drop should not be broken up too much or the fine débris will be carried into the final hanging drop and injure the preparation.

The dry blood method has the great advantage of convenience and is the method employed by nearly all laboratories for the Widal reaction for the diagnosis of typhoid. Although the dilution by color is relatively inaccurate because of differences in hemoglobin content it is sufficiently accurate for practical purposes.

Technic of Microscopic Reaction (Widal Reaction).—Preliminary dilutions of the blood or serum are prepared one-half the strength of the final dilutions desired. A loop of each dilution is placed on separate cover-slips, as well as a loop of the diluent. Then at the side of each drop is placed a loop of the culture. The two drops on each slip are then mixed with the platinum wire. This results in a doubling of each of the blood dilutions. Hollow-ground slides, the edges of the concavities having been smeared with vaselin, are warmed and placed over the cover-slips so as to seal the drop in the concavity. Warming the slides softens the vaselin so that it spreads better. The seal must be complete or the drops will dry up. Another sealing method is to place a drop of cedar oil on each corner of the cover-slip. In place of the sticky cedar oil, a mixture of albolene and paraffin may be used. On pressing the hollow-ground slide against the cover-slip, the oil spreads and gives a complete seal. The slide is then turned face up and the reaction observed under the No. 6 objective (magnification about 500 diameters). The drops must hang freely, that is, they should not be too large so that there is contact with the hollow of the slide. The drop containing diluent and culture alone is the "control." This must be carefully observed to eliminate errors due to spontaneous agglutination and as an index of the motility when a motile bacterium is employed.

A broth culture incubated for eighteen hours is usually employed in the microscopic reaction. When employed for the diagnosis of disease a "standard" "culture" is employed, that is, one of known agglutinability, otherwise the results will not be comparable. Such a culture should be one that, due to cultivation on artificial culture media, has reached a constant level of ease of agglutinability.

The beginner is apt to use too heavy a culture. The broth culture may give more delicate and sharper results when diluted by adding sterile broth.

The slides may be kept at room temperature, 20° C. or at incubator 35° C. The reaction proceeds more rapidly at the higher temperature.

If the reaction with a motile bacterium, B. typhosus for instance, is observed through the microscope in a hanging drop a formation of clumps is seen which, if it takes place rapidly, reveals the reaction almost completed at the first glance, that is, most of the bacilli are in loose clumps and nearly or altogether motionless (Fig. 81). Between the clumps are clear spaces containing few or no isolated bacilli. If the reaction is a little less complete a few bacilli may be found moving slowly between the clumps in an aimless way, while others attached to the

clumps by one end are apparently trying to pull away, much as a fly caught in fly-paper struggles for freedom. If the agglutinating substances are sufficiently present, but still less abundant, the reaction may be watched through the whole course of its development. Immediately after mixing the blood and the culture together it will be noticed that the bacilli move more slowly than before the addition of serum. Some soon cease all progressive movement, and it will be seen that they are gathering together in small groups of three or more, the individual bacilli being still somewhat separated. Gradually they close up the spaces between them and clumps are formed. According to the completeness of the reaction, either all of the bacilli may finally become clumped and immobilized or only a small portion of them, the rest remaining freely motile, and those clumped may appear to be struggling for freedom. With blood containing a large amount of agglutinating substances all the gradations in the intensity of the reaction may be

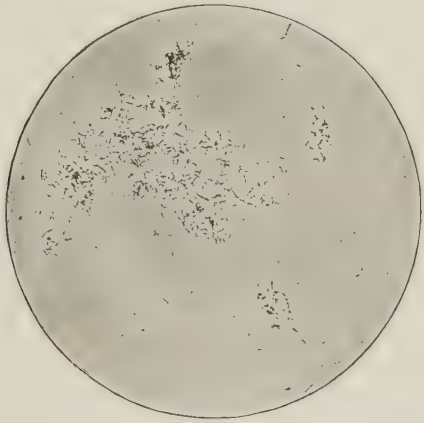


FIG. 81.—Widal reaction. Bacilli gathered into one large and two small clumps, the few isolated bacteria being motionless or almost so.

observed from those shown in a marked and immediate reaction to those appearing in a late and indefinite one by simply varying the proportion of blood added to the culture fluid. In general practice dried blood is more commonly used than serum because of its convenience. Serum is more desirable from the standpoint of accuracy of dilution and absence of débris. With careful standardization by color the dilutions made from dried blood are sufficiently accurate for routine diagnostic work. It is not altogether reliable to make only a low dilution. With a small percentage of blood or serum lysis may be marked and this may on casual examination give an apparently negative result. If higher dilutions had been made lysis would have been less and agglutination if present would be evident. In any event a careful comparison with the control will indicate that lysis has occurred and will prevent error from this source.

Wilson prepared a set of sketches to aid the student in making his

observations. As will be seen from the sketches of the cross-sections of hanging drops (Figs. 83, 85 and 87), such drops must have sufficient depth to show several focal planes. Fig. 82 shows the upper plane of the "control" drop; typhoid culture without blood solution or serum.

FIG. 82.—Microscopic field, showing the top of a hanging drop in a normal typhoid culture.

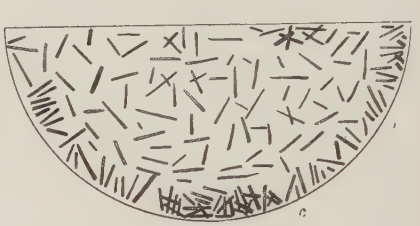


FIG. 83.—Microscopic field, showing a cross-section of the drop in Fig. 82.

Fig. 83 shows the cross-section of this drop, and indicates the microscopic pictures that will appear at different levels. These figures show the tendency of the bacilli, mostly non-motile, to gather at the edges and at the convexity of the drop. In this drop most of the bacilli would be actively motile.

FIG. 84.—Microscopic field, showing the top of a drop with the typhoid reaction.

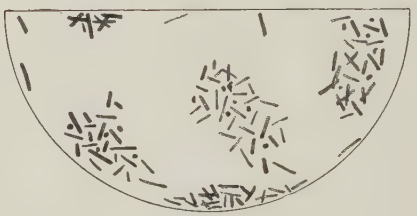


FIG. 85.—Microscopic field, showing a cross-section of the drop in Fig. 84.

Figs. 84 and 85 show the pictures obtained with a positive reaction. The edges and convexity of the drop are practically free of bacilli.

Motility is lost and the clumps are practically quiescent. The clumps tend to settle toward the convexity of the drop.

Fig. 86 and 87 show the appearance that may be noticed when blood from other than a typhoid infected person is used. The gathering at the edges and convexity of the drop is similar to that seen in the control. The blood solution contains considerable detritus especially in the lower dilutions. The figures show the tendency of the bacilli to adhere to or to become entangled in this detritus. These adherent bacilli frequently still show motility and most of the isolated bacilli are motile.

Fig. 86.—Microscopic field, showing the top of a drop of culture with reaction not due to typhoid.

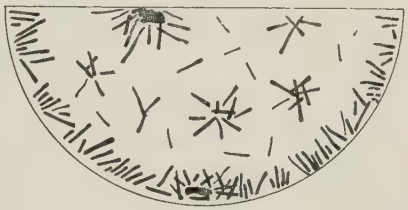


Fig. 87.—Microscopic field, showing a cross-section of Fig. 86.

Pseudo-reactions.—An exaggeration of the above described pseudo-clumping may develop if, through error, the concentration of the blood solution is too great. The pseudo-clumping especially after one-half hour tends to become very marked and isolated motile bacilli become much lessened in number. With poor light such pseudo-clumps may be mistaken for true agglutination. As a rule careful examination will show considerable motility among the adherent bacilli. If partial drying of the drop occurs, pseudo-clumping and considerable loss of motility may occur, due apparently to substances on the cover-slips. With all pseudo-reactions, however, bacilli can be found, usually in considerable numbers at the edge and in the convexity of the drop.

Technic of the Macroscopic Tube Method.—The suspension employed may be a broth culture or a suspension in saline of the growth from an agar slant or other solid media. In the case of bacteria which autolyze easily (meningococci and gonococci) the suspensions should be heated to 56° C. to destroy the autolytic ferment Such suspensions may be preserved for future use by adding a preservative, such as 0.25 per cent. carbolic acid. The suspension should not be too dense. A very slight clouding gives the sharpest results. Broth cultures to which 0.1 per

cent. of formalin has been added make very satisfactory antigens, especially of bacteria of the typhoid-paratyphoid group. As a rule, dilution of a broth culture by an equal volume of salt solution containing 0.2 per cent. of formalin gives a very satisfactory density. With B. abortus (melitensis) a freshly prepared saline suspension is most satisfactory. Freshly prepared suspensions of washed streptococci from young cultures are to be preferred. It should be emphasized that for comparative work antigens of like turbidity should be employed. Prepare the initial serum dilutions ten times more concentrated than will be the desired end dilutions. Place 0.1 c.c. of each dilution in a small chemically clean test-tube and 0.1 c.c. of saline in another for a control. To each of the tubes add 0.9 c.c. of the bacterial suspension, shake the tubes and incubate in the water-bath or incubator at 37° to 55° C. With some bacteria (meningococci) incubation at 55° C. over night gives the best results. With typhoid-paratyphoid group incubation for two hours and holding in the refrigerator over night gives the cleanest-cut results. Naturally readings may be made sooner if haste is an item. The procedure used must fit the character of the organism and the purpose in view.

If agglutination takes place it is shown first by a change in the appearance of the suspension. Instead of the uniform opacity there develops a ground-glass appearance. If one uses a lens one can see very fine clumps. As the reaction proceeds the clumps become more easily visible. If after incubation for two or three hours the tubes are allowed to stand, preferably in the ice-chest overnight, the clumps fall to the bottom of the tube. If the reaction is complete the supernatant fluid will be clear. In settling, the agglutinated bacteria form a broad film over the bottom of the test-tube, whereas the sedimentation of non-agglutinated bacteria results in a compact button-like sediment.

A satisfactory way to check the character of the sediment is to revolve the tubes between the palms of the hands. The swirl of the clumps leaves no doubt as to the character of the sediment. Occasionally with non-motile bacilli, either because the serum is of low titer or because the bacilli agglutinate badly, the fluid may be clear, but slight or no coherent clumps will form. Such results may be due to specific agglutination but they are difficult to read with any degree of certainty.

The control tube should always be carefully inspected to exclude spontaneous agglutination.

Comparison of Tube and Microscopic Slide Methods.—The reaction is the same in both and one is as reliable as the other. The ice-box readings (macroscopic) are apt to be higher than those obtained by microscopic examination. For diagnostic examinations in which haste is necessary and small amounts of serum are available, as in typhoid fever, the microscopic method is preferred. When a delay of twenty-four hours is no handicap and the serum is abundant as in tests for glanders in horses the macroscopic tube-test is generally used. Dead cultures are more frequently used in the macroscopic method because the motility here is of no importance.

Dreyer Method of Macroscopic Agglutination.—The basis of this method is the employment of a standardized antigen, a formalinized broth culture. This makes more uniform and comparable results possible. The method was suggested primarily as a means by which the rise or fall of agglutinins could be accurately determined. Thus, Dreyer believes typhoid or paratyphoid fever can be differentially diagnosed in the vaccinated because the rise or fall of the infection agglutinins are marked within short periods, whereas the agglutinins due to vaccination will be relatively stable during a similar period. To be able to determine a rise or fall with accuracy it is obvious that a standardized antigen must be employed.

For further details, including the methods Dreyer uses in expressing the results, reference is made to the article by Davison.[1] The use of agglutination antigens thus standardized is preferable in determining the curve of agglutinin response to vaccines in man or in animals, especially when the agglutinin response is used as a presumptive indication of the antigenic value of the vaccine.

The Macroscopic Slide Method.—This method allows a rapid identification of colonies from plates inoculated with suspected material, such as feces, throat cultures, etc. Coca[2] was apparently the first to use it practically. He applied it in the identification of cholera vibrios. Krumwiede[3] has employed it extensively in the identification of members of the typhoid-paratyphoid group of B. dysentery varieties and to the identification of meningococci from naso-pharyngeal cultures. Its great practical value is the rapidity with which a large number of cultures can be tested. A highly potent serum, whose specific and group agglutinating strength is known, should only be used or false positive results will be obtained. The group action of the serum should be low.

The method is as follows: A loopful of saline solution (as control) and one of the highly potent specific serum in low dilution are placed on one slide and a sufficient amount of the suspected colony to give a slight turbidity is added to each. Flocculation begins in the serum almost at once if the organism tested is specific. It is hastened by stirring A negative reaction is not exclusive, as relatively inagglutinable strains may be encountered, although with highly potent serum there is nearly always some evidence of a reaction. Colonies apparently typical, but not distinctly agglutinable, should be fished for further identification. Colonies giving a definitely positive reaction should also be fished for verification, unless experience has shown that the serum used does not give false positive results with allied types. Krumwiede[4] devised a dropping bottle which saves considerable time in placing the drops of diluted serum and saline on the slide.

Acceleration of Reaction by Shaking.—Dineur (1897) observed that if an emulsion of bacteria to which a specific antiserum is added be

[1] Jour. Am. Med. Assn., 1916, **66**, 1297.
[2] Bull. Manila Med. Soc., 1910, **2**, No. 1.
[3] Jour. Am. Med. Assn., 1917, **69**, 358; Jour. Infect. Dis., 1918, **23**, 275
[4] Jour. Immunol., 1920, **5**, 155.

gently shaken the clumping is very much increased. This phenomenon has been rediscovered by workers in different laboratories and from time to time practical applications have been suggested. Garrow's agglutinometer[1] was designed to rotate the slides carrying the mixtures of serum and bacteria, thus markedly accelerating the appearance of the macroscopic agglutination. The same phenomenon is noted, if in the macroscopic slide method the mixture of serum and homologous bacterium is stirred with the platinum loop after mixing. We have taken advantage of the phenomenon in pneumococcus typing, accelerating the reaction by gentle shaking. Noble[2] recently has called attention to the effect of shaking. He recommends the mixture of 0.1 c.c. of serum dilution and 0.1 c.c. of bacterial suspension in small test-tubes, shaking for two minutes and then adding saline to 1 c.c. The concentration of serum and of bacteria in the volumes mixed should be of such concentration so that after dilution to 1 c.c. the serum and bacterium content will be the same as in the standard tube method. It should be emphasized that the accelerating effect of shaking is most marked when shaking follows immediately after mixing. The shaking influences the physical phase of agglutination but not appreciably the binding of agglutinins.

Agglutinin Absorption.—Application and Interpretation.—The subject divides itself into two phases: (*a*) The use of agglutinin absorption as a means of bacterial identification; (*b*) as a possible diagnostic procedure applicable to patients' serums.

A detailed discussion of this reaction in all its phases would take more space than could be given in this place. Krumwiede, Cooper and Provost of this Laboratory have carried out an extensive study of the whole subject and the following gives a brief outline of their technique and conclusions.[3]

Application in Identification and Classification.—The fundamental requirements are proper reagents and proper standardization of these reagents. The general facts concerning the preparation of agglutinating antiserums have been given. It is desirable that the antiserums be as free as possible of normal and of group agglutinins,[4] that they be of high titer but not excessively high. For all careful experimental work[5] it is highly desirable that the worker prepare his own antiserums rather than use stock antiserums from other sources, likewise no antiserum should be used for experimental work for which the worker does not possess the actual culture employed in its preparation. The substitution of a like culture is valid in direct proportion to the degree to which the similar culture has been demonstrated to be *identical* in every way with the serum culture. What follows implies the use of monovalent antiserums and the absorption or non-absorption of the specific agglutinins as a criterion. Variations from this procedure are commented on later.

[1] Lancet, 1917, **1**, 262. [2] Jour. Bacteriol., 1927, **14**, 287.
[3] Jour. Immun., 1925, **10**, 55.
[4] See exceptions under Group and Specific Components.
[5] Those absolute requirements may be modified in routine.

Standardization of Absorbing Dose, Etc.—The first standard to be determined is the minimum dose of the homologous or serum culture which when added to a standard amount of its antiserum, under appropriate conditions, will remove all the agglutinins. Various methods may be employed to measure the absorbing dose, counts, opacity, weight or volume. The use of volume is on the whole the most convenient, especially with antiserums for bacteria of high antigenic value. For instance with B. typhosus an antiserum of a titer of 1 to 10,000 or better is common, whereas with meningococci the obtainable titer may not be over 1 to 200 to 300. In the latter instance the volume of the absorbing dose would be too small to handle. Therefore in this case a fraction of a measurable volume can be employed. Agar or broth cultures may be employed. If from agar the cultures are suspended in saline and this suspension or broth cultures are centrifuged in calibrated centrifuge tubes until the bacteria are solidly packed. The amount is noted and the supernatant fluid decanted. To the packed organisms are added antiserum and saline to give the desired serum dilution and proportion of packed organisms to diluted serum. To compensate for the saline in the bacterial mass a correction of about 10 per cent. is adequate. Thus if the bacterial mass measured 0.4 c.c. its actual volume would be considered as 0.36 c.c. If a proportion of bacteria to serum dilution of 1 in 10 were desired the total volume required would be 3.6 c.c., which less the volume of the bacilli would give 3.24 c.c., the total fluid needed. If a dilution of serum of 1–10 is to be employed, 0.324 c.c. of serum is added and the volume brought to 3.6 c.c. by the addition of saline.

(Mass -10 per cent.) $\times$ absorption dose factor = total volume.

(Mass -10 per cent.) $\times$ (absorption dose factor -1) $\div$ dilution factor = serum required.

The bacterial sediment is broken up and an even mixture of bacteria and diluted serum obtained. Incubate at from 37° to 45° C. for two to three hours, shake at intervals if settling is marked, place on ice overnight and centrifuge to clarify diluted serum.

Dilutions are made and bacterial antigen added to give the appropriate dilutions (see below). A control of untreated diluted serum should have been carried through the same manipulation as a control on deterioration. The minimum absorbing dose is thus determined by trying different proportions of bacteria and dilute serum as outlined above. To test the absorptive capacity of other cultures twice this volume, as above determined, is satisfactory as a rule. This suffices to exclude minor variations and compensates for the moderate errors inherent in the method. Close titration and comparison may be necessary at times. The agglutination antigens and the temperature and time allowed for reaction in all tests naturally should be such as to give comparable results in any series of tests.

General Consideration.—As criteria of likeness or identity the following will serve as a general basis. If culture A will reduce the agglutinin content of antiserum B so that it will not agglutinate its homologous

culture in dilutions of less than 5 per cent. of its original titer, the two bacteria are presumptively similar. This however is not a criterion of complete absorptive likeness as the *reciprocal test* may or may not be positive. That is bacterium B may or may not be able to absorb the agglutinins for bacterium A from serum A. By such reciprocal tests interrelationships, especially the dominance or subsidiary relationships (groups, subgroups) are determinable. Partial absorptions are relatively common and may be employed as a means of determining relationships. Such partial absorptions show the necessity of testing in low dilutions for final criteria.

The absorption by two or more cultures of the same group agglutinins is not a criterion of similarity as the cultures may not be able to absorb the same specific agglutinins. The group-absorption method, if employed, has only suggestive value. The use of polyvalent serum for absorption work is not to be advised, even if after absorption each of the serum strains are used for test. The method is unwieldy and errors may be introduced because of the multiplication of factors. If the serum cultures are not tested the method becomes simply one of group agglutinin absorption. Zone phenomena may lead to confusion. These are caused by products of the media and autolysis. They are minimized if the original suspending fluid is eliminated by preliminary centrifuging (see above). They tend to become exaggerated when absorption is done by adding successive dosages of bacteria. Differences in agglutinability among cultures of the same variety may at times be very marked. This must be considered in interpretation or error will occur.

Constancy of Absorptive Capacity, Etc.—The absorption capacity of a bacterial culture, as such, shows in most instances a marked tendency to constancy. A culture however is not necessarily homogeneous but may be looked upon rather as a race or composite. With paratyphoid bacilli, for instance, two components may be obtained from practically every culture, which may be termed group and specific respectively. The specific component is not or only slightly agglutinated by the antiserums for heterologous but closely related varieties, whereas, the group component is strongly agglutinated by such serums. If antiserums are prepared for each of these fractions it will be found, that neither component will absorb all the agglutinins from the antiserum of the other component. Another variant that may be encountered may be termed a degraded variant. This agglutinates poorly and in compact fine clumps and is unable to absorb all the agglutinins from an antiserum prepared by injection of the original undegraded culture. An antiserum prepared for the degraded variant may contain agglutinins for the parent culture which the degraded variant is unable to absorb. When the antiserum is absorbed by a related culture the agglutinins for the degraded variant may be removed showing that degradation may lead to loss of specificity. Colony variants are not uncommon, but these may or may not be associated with serological variation. Mucoid variants may be observed with paratyphoid cultures and these

may show somewhat marked antigenic and agglutinative absorptive differences from the non-mucoid variety of the same culture. The loss of the mucoid character is associated with a return of all the characters of the type. The so-called "rough" colony type[1] may or may not be associated with absorptive variation. The variants mentioned develop under what might be termed "normal" conditions of cultivation, and naturally if a culture becomes dominated by or becomes purely one variant in constitution this would lead to difficulties in its identification or classification. All the above variants of B. paratyphosus have been isolated directly from original infectious material. Other and more extreme variants have been described as having been induced by abnormal conditions of cultivation. Whether such more extreme variants occur under ordinary conditions in cultures from infectious material or in stock cultures maintained under appropriate conditions remains to be seen. Many instances have been reported of supposed transmutations of type, but the evidence is not acceptable. Recently, however, Griffith[2] has claimed to have converted degraded strains of one type of pneumococcus into a different type by inoculating the degraded strain and massive doses of killed virulent organisms of another type subcutaneously into mice. The experiments are apparently well controlled, but the work waits confirmation. Griffith offers direct experimental evidence, whereas most other reports have been based upon indirect evidence. Although we see no flaw in his report, we are not convinced.

The argument that if an epidemic of a disease were due to a microorganism, the strains isolated would belong to a definite type, is apparently weakened by the observations on variation. However, if, for example, B. influenzæ were the causative agent in influenza, variations might occur, but is there reason to believe from the expermental data available that such variations would be so rapid and frequent as to give the appearance of the marked heterogeneity which has actually been found? Opinions to the contrary are largely hypothetical, and assume that variation is as active and rapid under natural parasitism as under more artificial laboratory conditions. The proof of this waits on future direct observations. The reverse of the above argument, that if all or nearly all the cultures obtained are alike, the organism must be the causative agent is probably too extreme. It is within the range of probabilities that in an outbreak of hog cholera the great majority of the animals might show secondary infection by the same variety of B. suipestifer.

Diagnostic Application.—Castellani had advocated the application of the absorption method as a means of differential diagnosis. Thus given a case of enteric fever, his argument might be stated thus: The patient's serum agglutinates T. A and B;* absorb with each of these

[1] Considerable confusion and apparent differences of opinion have arisen due to the use of the term rough or the symbol R to designate degradation, loss of virulence, serological change, etc. We are employing the term to describe the colony only.

[2] Jour. Hyg., 1928, vol. **27**, No. 2.

* Abbreviations for B. typhosus, B. paratyphosus A and B.

varieties and test the absorbed serum against each of the types. If A and B absorb the agglutinins for themselves only but not for T., but T. absorbs all the agglutinins for T. A and B then T. is the infecting bacterium. If B removes the A and B agglutinins only and T. removes the T. agglutinins only then a double infection by T. and B exists. This argument is based on the assumption that T. A and B are the only bacteria causing enteric fever. As we now know other varieties do, and in both of the above instances the infecting bacterium may have been C. Park had called attention to this possible source of error. Other factors may enter and introduce error. A striking example of the inherent fallacy of Castellani's conclusion is shown by the following.[1] The antiserum was from a rabbit injected with a culture of a B. paratyphosus variety which is designated as No. 8, and the absorption was carried out with the cultures as designated.

RABBIT SERUM—ABSORPTION OF B. PARATYPHOSUS NO. 8 ANTISERUM.

Culture used for agglutination.	Titer before absorption.	Results of absorption with cultures.					
		B. para 1.	B. para 2.	B. para 3.	B. typh.	B. para 6.	B. para 8.
B. para 1 .	3200	+	−	−	−	−	+
B. para 2 .	800	+	+	+	−	−	+
B. para 3 .	1600	+	+	+	−	−	+
B. typhosus .	3200	−	−	−	+	−	+
B. para 6 .	1600	−	−	−	−	+	+
B. para 8 .	3200	−	−	−	−	−	+

+ = Absorption of agglutinins for culture in left hand column.
− = Absorption negative.

If the first three cultures only had been tested, the conclusion according to Castellani's argument would have been that B. paratyphosus No. 1 was the infecting bacterium. Adding B. typhosus the results according to Castellani would prove a double infection, or adding B. paratyphosus No. 6 a triple infection. As a matter of fact the bacterium stimulating these agglutinins was B. paratyphosus No. 8.

This definitely shows that without the specific stimulating culture we have no actual standard. If therefore the agglutinins present in blood of a case showing enteric fever symptoms are actually due to the stimulation by a typhoid-paratyphoid variety (which may not be the case) absorption may at most give suggestive results, but only when every possible bacterial variety causing enteric fever in any vicinity is included in the test.

Diagnostic but not Specific Agglutination.—This curious phenomenon occurs sometimes. Thus, Wilson,[2] in 1909, showed that the serum from cases of typhus fever has considerable ability to agglutinate

[1] Krumwiede, Cooper and Provost: See reference.
[2] Jour. Hyg., 1910, **9**, 316; 1920, **19**, 115.

certain intestinal bacteria. Later Weil and Felix[1] isolated B. proteus cultures from urine which agglutinated in typhus serum. They have studied various proteus cultures and have selected a variety which they term B. proteus X 19, as most satisfactory for use. Numerous hypotheses have been advanced to explain the appearance of these agglutinins. Some have drawn the conclusion that B. proteus has some etiological relationships to the disease. There is little evidence in favor of such a conclusion. Wilson has observed similar peculiar agglutination reactions with the serum from cases of meningitis. A salt solution suspension from an eighteen-hour agar slant is the best antigen for this test.

Agglutinins for Other than Microbal Cells.—Hemagglutinins, that is, agglutinins for red cells, may be normally present, or specific hemagglutinins may be produced by the injection of red cells. Goat serum normally contains agglutinins for rabbit corpuscles. Iso-agglutinins for the red cells of other individuals of the same species are encountered. This is of extreme importance in the transfusion of blood from man to man.

Conglutination.—Bordet and others described a peculiar auxiliary substance in beef serum which they term "conglutinin." A resulting test known as the conglutinin reaction has been used to some extent.[2]

[1] Wien. klin. Wchnschr., 1916, **29**, 33 and 873.

[2] Cent. f. Bakt., 1909, **49**, 26; Ann. Inst. Pasteur., 1906, **20**, 467; Ztschr. f. Immunol., 1916, **25**, 219.

CHAPTER X.

ANTIMICROBAL OR ANTIPROTEIN SUBSTANCES.
(CONTINUED.)

PRECIPITINS.

PRECIPITINS are antibodies which, when added to a clear bacterial extract, cause a precipitation. In 1897 Kraus noted that if the serum of an animal immunized against B. typhosus is added to bacterial-free filtrates of broth cultures the mixture first became turbid, which change was followed by precipitation. Similar results were obtained with other antisera and their homologous bacteria.

Nature of Precipitins.—The properties of precipitins are very similar to those of agglutinins. They are fairly resistant, but are gradually destroyed by heating to 60° to 70° C. Ehrlich and Bordet consider the precipitin antibody as having the same structure as the agglutinins. Ehrlich classes it as an antibody of the second order. The loss of activity of precipitins according to this conception would be due to the deterioration of the more labile zymophore group, resulting in the formation of "precipitinoids." Precipitins are specific within certain limits.

Mechanism of the Precipitin Reaction.—If we look upon the precipitin antigen, that is, the clear solution of microbal constituents as simply a suspension of invisible molecules as contrasted with microscopically visible microörganisms in an agglutination antigen, the mechanism must be similar to that of the agglutination reaction. The known facts and theories of the mechanism of one reaction apply in general to the other.

Prezone Phenomenon.—This phenomenon occurs with low dilutions of some precipitating sera or with too concentrated antigens and is directly analogous to that observed in the agglutination reaction.

Specific (Major), Group (Common or Minor) and "Normal" Precipitins.—During immunization both specific precipitins for the homologous bacterium as well as group precipitins for closely related types are produced. The reasons for this phenomenon are the same as given under agglutination. Normal precipitins for bacterial extracts are less commonly encountered than are normal agglutinins. Horse serum is most likely to contain them.

Development of Precipitins.—Precipitins are not commonly demonstrable in the serum of infected individuals. This antibody is usually present in appreciable amounts only after a prolonged and intensive stimulation due to natural infection or artificial immunization. The precipitinogen, that is, the stimulant to its production, may be live or dead bacteria or solutions of their substance obtained by extraction or autolysis.

Practical Applications of Precipitin Reaction.—The precipitin reaction has only a limited application in the identification of bacteria in culture and body fluids and discharges. It is less easily applied than the agglutination reaction, because of the necessity of preparing a clear bacterial extract. Another disadvantage is that the range of precipitin action of an antiserum is relatively low when ordinary extracts are employed. With the purified soluble specific substance the reaction is more delicate and a wider working range of dilutions becomes available. (See below, Technic.) At present the reaction is limited in practical work to the differentiation of the types of pneumococcus. An important application is the determination of the presence and variety of bacterial substance in excretions, exudates or lesions. Thus, in lobar pneumonia considerable soluble products of the pneumococcus are found in the lung, in the sputum, in the blood and thence in the urine. Advantage is taken of this fact by using the precipitin reaction to determine the type of infecting pneumococcus. Likewise, if the sputum is injected intraperitoneally into the mouse the precipitin reaction may be similarly applied to the peritoneal exudate. In the case of anthrax infections in animals extracts of the lesions give a precipitin reaction with immune anthrax serum, thus permitting a diagnosis. (For details of methods see under these diseases.)

The precipitin reaction with the serum of the infected host, because of the absence of appreciable amounts of precipitin, has been applied practically very little in the diagnosis of disease. The serum of horses infected with glanders usually gives a precipitin reaction, and the method has been used for diagnosis. The results are less reliable than those obtained with the agglutination or other serological reactions.

Technic of Reaction.—A highly immune serum must be available and the range of specific and group as well as normal precipitins must be known. The antigen may be prepared from a lesion, exudate or excretion (see Pneumococcus and Anthrax) or from agar or broth cultures. If broth cultures are incubated for several days or longer the clear broth obtained by filtration or centrifuging will give a very satisfactory antigen. With the pneumococcus precipitable substance will be found in broth after a short incubation of four to six hours. Similar results may be obtained by suspending and extracting the bacteria from agar cultures in salt solution or distilled water. Or the suspension may be made in decinormal NaOH solution and boiled. This, when neutralized and cleared, gives a very satisfactory reagent (antigen). Bacteria other than the acid-fast types can be dissolved in alkaline hypochlorite solution (antiformin). Taking advantage of these facts, we have devised a rapid method of preparing a concentrated reagent by adding antiformin to a concentrated suspension of bacteria, boiling, precipitating with alcohol, dissolving the precipitate in saline and clearing by centrifuge.[1] The precipitate that occurs promptly after the addition of the alcohol gives the best reagent. Pneumococci may be dissolved by the addition of bile.

[1] Krumwiede and Noble: Jour. Immun., 1918, 3, 1.

The serum undiluted and diluted 1 to 5 to 1 to 25 or more is added in 0.2 c.c. amounts to small test-tubes. To this is added a similar amount of antigen. The tubes are then placed in the water-bath or incubator at 37° to 55° C. If the reaction occurs it is first noted by the appearance of turbidity. If the reaction progresses, small floccules separate which increase in size and fall to the bottom of the tube, leaving the supernatant fluid clear. The tubes may be incubated any arbitrary time suitable to the purpose in hand. With the reactions of lesser degree storage on ice overnight aids in making comparative readings. The prezone phenomena may be noted if the antigen is too concentrated.

The following show the range of specific and group action with highly immune sera, using the usual types of extract antigen (reagent).

PRECIPITIN REACTIONS—PNEUMOCOCCUS SERA.

Pneumococcus antigen.	Time, hours.	Serum types and dilutions.								
		Type I.			Type II.			Type III		
		1–1	1–4	1–9	1–1	1–4	1–9	1–1	1–4	1–9
Type I	1	++	+	±	−	−	−	−	−	−
	2	+++	++	+|	±	−	−	−	−	−
Type II	1	−	−	−	++	++	+	−	−	−
	2	±	−	−	+++	+++	++	−	−	−
Type III	1	−	−	−	−	−	−	+	−	−
	2	−	−	−	−	−	−	+|	±	−

Antigen used = 0.2 c.c. Serum solution = 0.2 c.c.
+++ = profuse precipitate. ++, +|, +, ±, = decreasing amounts of precipitate.

INFLUENCE OF DILUTION OF ANTIGEN ON CROSS-REACTIONS.

Organism.	Serum dilution (anti-typhoid).	Antigen diluted 1–10			Antigen diluted 1–80.		
		One-half hour.	One hour.	Ice-box.	One-half hour.	One hour.	Ice-box.
B. typhosus	1:1	++	++	++	+	+	+
	1:6	++	++	++	+	+	+
	1:12	+	+|	+|	+	+	+
B. paratyph "A"	1:1	C	±	+	−	−	−
	1:6	−	±	+	−	−	−
	1:12	−	±	+	−	−	−
B. paratyph "B"	1:1	±	±	+	−	±	±
	1:6	−	Sl. C	±	−	−	±
	1:12	−	−	±	−	−	±
B. sanguinarium	1:1	+|	+|	++	+	+	+
	1:6	+|	+|	+|	+	+	+
	1:12	±	+	+|	+	+	+

Sl. C. = slightly cloudy. C = cloudy. ± = slight flocculation. + = distinct flocculation. +| = flocculation more marked. ++ = heavy precipitate. Antigen 0.2 c.c. and serum dilution 0.2 c.c. used.

The second table is given to show the influence of the concentration of antigen on the degree of cross-reaction. The precipitation ceased after dilution to 1 to 100. The last strain of fowl origin is included to show the extremes of cross-precipitation that may be encountered. This type tends also to cross-agglutinate, very markedly with typhoid antiserum. In this instance, however, the serum agglutinated B. typhosus in a dilution of 1 to 10,000, B. sanguinarium only to one-seventh of this dilution.

The delicacy of the precipitin reaction can be increased by floating the antigen or reagent over the serum. In this case the reaction is shown by the development of a ring at the line of junction. This is feasible only when the serum is not diluted or diluted slightly and the antigen has a lesser specific gravity.

With the purified soluble specific substance (see under Agglutination) as precipitating reagent much more specific reactions may be obtained than with the cruder extracts. The reasons for this are given in the discussion referred to.

The Nature of the Precipitin Antigen or Reagent.—As was outlined under Agglutination, two bacterial constituents must be considered. The protein content is antigenic and reactive; the carbohydrate content is reactive, but not antigenic. The former underlies common or group reactions; the latter specific reactions, as a general rule. In other words, the specificity of the cruder extracts depends on the presence of the specific substance. This explains why very satisfactory precipitating reagents may be prepared by the alkaline hypochlorite method. As in a true sense the carbohydrate is not antigenic, solutions of this substance or extracts, which are crude solutions, should be termed precipitating reagents. In the preparation of precipitating antiserums the antigen of choice is the whole unaltered organism if the greatest degree of specificity is to be obtained. Students commonly get the idea that an extract must be used for the injection of animals possibly through the confusion of the use of antigen, for both stimulating and reacting substances.

Other Antibodies in the Precipitate.—Agglutinins, bacteriolysins, complement-fixing substances and protective antibodies are carried down with the precipitate. These can be dissociated to some extent from the precipitate by extraction with weak alkaline solutions. Gay and Chickering[1] attempted to utilize these facts as the basis of a practical method for the concentration of the antibodies in antipneumococcus serum.

Precipitin Absorption.—An absorption method similar to the agglutinin absorption method may be utilized as a means of differentiation between group and specific precipitins. The method has had only a limited application. Krumwiede and Cooper[2] have shown that with extract reagents the specificity is less than with the agglutinin absorption method. That is, precipitin absorption may fail to differentiate between two closely related bacterial types which are easily differentiated by agglutinin absorption.

[1] Jour. Exp. Med., 1915, **21**, 389; also Weinstein: Jour. Immun., 1918, **3**, 17.
[2] Jour. Immunol., 1920, **5**, 547.

Precipitins for Other than Microbal Proteins.—Tchistovitch and Bordet showed that the injection of alien serum would result in the production of precipitins for the injected serum. Similarly, precipitins can be prepared for vegetable proteins. Further observations revealed that this reaction had species specificity, that is, the reaction occurred quantitatively highest with sera from individuals of the same species as used for injection. The degree of group reaction with sera of other species depends on the closeness of biological relationship of the species. The antiserum precipitin will react with the other albuminous secretions of the same species. By washing organs free of blood a certain degree of organ specificity seems demonstrable. One peculiar fact is worthy of note, however, the crystalline lens from all animals has the same protein content as determined by the precipitin reaction.

Practical Applications.—Although this is not within our subject a brief outline is given. Because of the marked species specificity the precipitin reaction may be employed to identify the source of blood stains for medico-legal purposes. It is also applied in the determination of the sophistication of foods.

Given a blood stain, presumably human, the first step is to prove that it is blood. If the blood cells have disintegrated, this is done by chemical means, preferably by the demonstration of hemin crystals. The extract of the stain in salt solution is used as the antigen in the test (see below). In the case of meats a salt solution extract of the meat, or, for instance, sausage, is made and used as an antigen with anti-beef antihorse, antidog, etc., sera. That giving reaction in dilutions commensurate with controls of known meat gives us our result. The antisera are obtained by several large injections of the species serum into rabbits. The technic of the reaction is based on the fact that even very dilute amounts of antigen are demonstrable. The antigen is diluted, therefore, not the serum. With very potent antisera even dilutions of 1 to 25,000 or more of the antigen will give a reaction. In the cases mentioned above an estimated initial dilution of 1 to 1000 is prepared, from which other dilutions can then be made. The antigen is then floated over the serum and incubation carried out at 37° C. for one to two hours. The appearance of a ring at the point of contact is a positive reaction. The reagents must be clear and many controls with other antisera and known antigens are necessary to exclude error.

Properties of Protein Antigen.—As contrasted with bacterial antigens they are very labile. Heating or chemical treatment renders the protein less or non-precipitable. If, however, an animal is injected with moderately heated protein a serum is obtained which precipitates the heated and the unheated serum as well as split products unaffected by the native serum precipitins. The species specificity is retained. Boiling for three to five minutes, however, reduces the specificity. These facts are utilized in testing heated meats for evidences of sophistication. It is claimed that certain types of chemical alteration destroy species specificity; for instance, iodized-beef protein, when injected, stimulates a precipitin not only for iodized-beef protein but for similarly altered proteins of other species.

CHAPTER XI.

ANTIMICROBAL OR ANTIPROTEIN SUBSTANCES.
(CONTINUED.)

BACTERICIDAL, BACTERIOLYTIC AND CYTOLYTIC ANTIBODIES.

IN 1888 Nuttal demonstrated that normal blood had a bactericidal or bacterium-killing property without regard to the presence of phagocytic cells. He showed that serous exudates contained similar substances and found that the bactericidal action was weaker after standing and was quickly destroyed by heating to 60° C. Buchner found the same property was possessed by serum obtained after the clotting of blood and called the active substance "alexin." Pfeiffer, in 1894, showed that if guinea-pigs were first immunized against the cholera vibrio and then injected intraperitoneally with living cholera vibrios there was a rapid swelling, granulation and dissolution of the bacteria. This process could be followed by removing portions of the peritoneal exudate with capillary tubes and examining it in stained smears. A normal guinea-pig would not show this phenomenon or at most slightly, but the phenomenon could be induced even with normal pigs if "immune" serum were introduced with the vibrios. The same result occurred if the serum was first heated to 60° C. This is known as the "Pfeiffer phenomenon." The active substance, therefore, was an acquired antibody, and, as he showed, was specific, acting only on the cholera vibrio. The hypothetical substance causing this action, Pfeiffer called "bacteriolysin," or specific bactericidal substance.

Bordet then showed that the phenomenon was due to the interaction of two substances, one the specific substance, increased during immunization, which was thermostabile (50° to 60° C.). He also showed that fresh immune serum may be actively bactericidal, but if this power were lost by standing or by heating it could be fully restored by the addition of fresh guinea-pig serum which possessed little bactericidal action by itself.

The Immune Body, Sensitizer or Amboceptor.—The terms bactericidal or bacteriolytic are used to denote whether the microörganism is only killed or killed and disintegrated. There is no evidence that we are dealing with two distinct antibodies. The term amboceptor was adopted by Ehrlich because he believed that the antibody was a cell receptor having two haptophore groups, one with an affinity for the microbe the other for combination with the third substance normally present in serum, which he called complement. Bordet objected to this term, and used sensitizer or immune body. (See below.) These antibodies resist

heating to 60° C., but are gradually destroyed by heating to 70° to 80° C. The amboceptor or immune body combines with the cell in the absence of complement. As with other antibodies, those produced during immunization have highly specific as well as some group or common action. They are also normally present to a varying degree.

The Complement or Alexin.—Complement or alexin is present in normal and in immune serum. Its quantity is not increased during immunization. It is the active substance in bacteriolysis. Its structure, according to Ehrlich, is very similar to that of a toxin possessing a haptophore group for combination with the receptor and an active or cytophile portion to which it owes its action. The latter portion is unstable, deteriorating when serum is left at room temperature for forty-eight hours or longer, and is destroyed by heating to 55° C. for one-half hour, and is very sensitive to acids and alkalis. The residual haptophore group Ehrlich terms a complementoid, and believes that it will combine with the receptor, thus preventing the action of active complement. Such evidence as is available indicates that the leukocytes are a source, if not the only source, of complement. How many varieties of complement exist is an open question. Bordet believes there is only one variety, whereas Ehrlich and others have advanced what they consider evidence to prove the existence of many varieties. Complement in its action resembles ferments very closely, but differs from ferments in being used up in the reaction.

Mechanism of Bacteriolytic Action.—According to Ehrlich's hypothesis, the amboceptor acts as a bridge for the action of complement. Bordet, however, believes that the antibody (sensitizer as he terms it) combines with the bacterial cell, sensitizing it to the action of the complement. In favor of such a conception is the fact that complement or alexin shows little if any affinity for the immune body in the absence of the homologous bacterium. If the amboceptor were simply a double haptophore bridge the complement should combine with the amboceptor. Here, again, we find differences in theoretical considerations based on hypotheses of quantitative chemical combination or on the more physical theories of colloidal adsorption. The combination of the antigen with amboceptor and complement is approximately quantitative as with other antibody combinations. The most important variation in quantitative relationship is seen in the fact that if the amount of amboceptor is increased less complement is needed, whereas if the amount of complement is increased less amboceptor is needed. The action of the alexin or complement on the cell sensitized by immune body would seem to be upon the cell envelope and stromata, making it permeable and liberating the protein content. Even though the mixtures of bacteria, homologous antibody and complement, be incubated for prolonged periods there is no evidence of proteolytic cleavage. Amboceptor and complement have different avidities in relation to temperature; thus amboceptor will combine with the bacterial cell even at a temperature just above the freezing-point, whereas complement will only combine with the cell-amboceptor complex at higher temperatures, its optimum

being at 37° C., at which temperature it also displays its highest activity. In the preceding discussion of the mechanism of bactericidal or bacteriolytic action only the combination of antibody and cell have been described. It should be emphasized, however, that immune body and complement are fixed by the substances in extracts of bacteria, even in the absence of demonstrable precipitation. (See Precipitins.) As was noted under agglutination specificity of antibody combination is due to the carbohydrate fraction of bacteria. This when obtained in pure solutions will specifically fix immune body and the complex will bind alexin.

Determination of Bactericidal or Bacteriolytic Action.—The activity of a serum may be determined by *in vivo* and *in vitro* methods. The Pfeiffer phenomenon in guinea-pigs is utilized for the former. The test is carried out by injecting a series of guinea-pigs intraperitoneally with a constant amount of culture and decreasing amounts of antiserum. The reaction is observed by removing portions of the peritoneal exudate to determine the degree of action. The smallest amount of serum yielding a positive reaction gives us the titer of the antiserum.

The test-tube method is usually carried out according to the technic of Stern and Korte. To a series of test-tubes add decreasing amounts of the inactivated (heated to 55° C. half hour) serum. To each tube then add 0.1 c.c. of fresh guinea-pig serum to supply a constant amount of complement. Add saline to bring the contents of each tube to constant amount, 0.5 or 1 c.c. To each tube add a small amount of dilute suspension or broth culture of the homologous organism. Incubate for three hours at 37° C., and then make poured agar plates of the contents of each tube and incubate and determine the relative number of colonies developing. Adequate controls must be made, salt solution plus culture controls, one to be plated at once, another after incubation, an amboceptor containing serum control of the lowest dilution without complement and a control containing 0.1 c.c. of the complement serum, each of the latter naturally have the culture amount added. The guinea-pig serum may be bactericidal itself. The sterility of each of the reagents must also be controlled.

It is obvious that with this method, even though we did not inactivate the serum, we are titrating the amboceptor content as we are adding complement to activate the amboceptor in the high dilutions. If we wished to determine the actual bactericidal value of native serum because of its content of immune body and natural complement we should carry out the test in a similar manner employing a constant amount of fresh active serum but decreasing the dose of culture. The number of organisms in the decreasing dose may be estimated by control plating. The largest number of organisms that is killed by this constant dose of serum is the measure of the serum.

The methods are relatively difficult to carry out and unsatisfactory results are common. Comparable results are difficult to obtain on successive tests. With the plate method irregularities are usually due to the variations in resistance of the culture or to guinea-pig serums which may be bactericidal to a varying degree. The tests are not applied

practically today for purposes of identification of bacteria or as a diagnostic procedure. The Pfeiffer reaction has lost its importance as a means of identification because the simpler agglutination reaction gives us the same information. Stern and Korte used the method as a diagnostic procedure in typhoid fever and found that the serum of a patient might be strongly bactericidal when the agglutination was low. The method is too complicated and the blood culture method is more easily applied in the earlier stages of the disease.

The methods are used primarily for experimental purposes to determine the response to injection of vaccines or to compare the bactericidal action of serums with the degree of action of other antibodies.

Prezone Phenomenon. — In the above *in vitro* tests it not infrequently happens that the larger amounts of serum show no bactericidal action. This might be explained as due to complementoids according to the Ehrlich hypothesis. The phenomenon may be due to colloidal reactions, as pointed out for other antibodies. Protective substances may be present which prevent amboceptor combination or complement combination (anticomplementary action) or both; these substances becoming insufficient to cause inhibition as the serum is diluted. The fixation of complement by the precipitin combination with any soluble antigen present has also been advanced as an explanation.

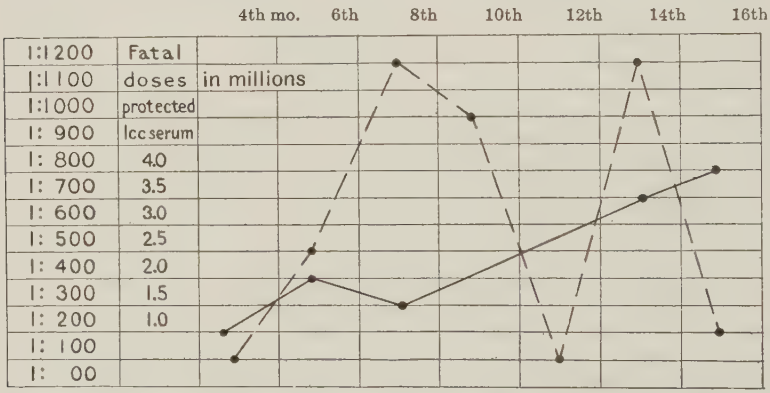


FIG. 88.—Relation of agglutinative to bactericidal power. Horse injected with culture of dysentery bacilli over a period of sixteen months.

—— —— —— Agglutination index.

———————— Bactericidal index.

· Test dates.

Relation between Agglutinating and Bactericidal Power.—In spite of proof to the contrary, the belief persists that there is regularly a parallelism between the agglutinating and the bactericidal strength of a serum. In Fig. 88 are recorded a number of comparative tests during a period of sixteen months. The experiment shows no definite relation between content of agglutinins and bactericidal antibodies. We have found in horses and other animals subjected to injections of a fixed type of pneumococcus that the agglutinating strength usually develops before

the bactericidal strength and that it decreases while the latter is still increasing. Both tend rapidly to decrease when animals are sick.

Lytic Antibodies for other than Microbal Cells.—Bordet showed that if the red cells of alien species were injected into an animal, antibodies were produced which will cause hemolysis of the cells. Although the cells were not dissolved the action of the antiserum resulted in a liberation of the hemoglobulin from the cell stromata, the latter remaining as shadow forms. It was soon evident that this action, as with bacteriolysis, was due to two substances: a hemolytic amboceptor, which resisted heating to 55° C., and whose production is stimulated by the injections and the complement, sensitive to heating to 55° C., which is a normal constituent of the blood. The amboceptor or immune body was found to be specific for the cells of the animal used for injections. Group antibodies may be present in less amounts, however, for the cells of allied species.

One species may possess natural hemolysins for the red cells of another species. Thus normal goat serum will hemolyze rabbits' red cells. Isolysins may be normally present in the serum of one individual for other individuals of the same species. This has to be considered in selecting donors for blood transfusion. Cytolysins may be produced for cells other than red cells.

Method of Eliciting Hemolysis and Titration of Reagents.—As this is of importance to us primarily in a specialized procedure, "complement-fixation," it is given in the chapter on that reaction.

CHAPTER XII.

THE COMPLEMENT-FIXATION REACTION.

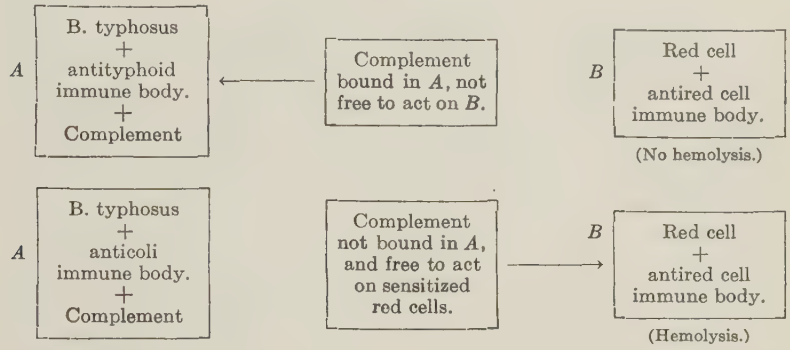
THE THEORY OF THE TEST AND ITS PRACTICAL APPLICATION.

In the preceding chapter it was seen that cytolysis results when two elements are present, immune body or amboceptor and alexin or complement. It was also shown that not only could antibacterial antibodies be produced, but also cytolytic antibodies active against other cells, red cells for instance. The antibody which is specific for red cells, and which sensitizes red cells so that complement will cause their hemolysis, is called hemolysin. This antibody is of special importance because the action results in a macroscopically visible change, and can, therefore be used as an indicator, as will be seen below. Two facts given in the preceding chapter should be remembered, immune body is relatively stable, resisting heating to 55° C. for one-half hour, alexin or complement is very labile, is destroyed by heating to 55° C. for one-half hour, and deteriorates rapidly at room temperature, somewhat more slowly in the refrigerator. Complement is not bound by free immune body, as suggested by Ehrlich's term amboceptor.

Bordet-Gengou Phenomenon. Complement- or Alexin-Fixation Reaction.—In the preceding chapter a method, *in vitro*, to determine the presence of bactericidal antibody was described. In this method inactivated antityphoid serum, in graded dilutions, was added to a constant quantity of typhoid bacilli and to this was added constant quantities of complement or complement-containing guinea-pig serum. After incubation no macroscopically visible change occurred, and it was necessary to resort to agar plating, to demonstrate whether or not the phenomenon of immune body-bacilli-complement had occurred; or, expressed another way, whether bactericidal action had occurred. Bordet and Gengou, in considering this difficulty, concluded that the phenomenon should be demonstrable in an indirect way, if an indicator could be devised which would macroscopically reveal the presence or absence of alexin or complement. They turned to the red cell sensitized with its antibody, the hemolysin as the indicator. Thus, if serum (inactivated) is added to typhoid bacilli, the deciding factor, whether complement will be bound, is the presence of specific antibody in the serum.[1] If antibody is present complement will be bound; if the indicator, red cells sensitized with homologous hemolysin be added, no hemolysis can take place, as the complement was bound by the sensitized typhoid bacilli (bacilli + immune body). If, however, a serum containing no antibody is added to typhoid bacilli complement will remain free, as there are no anti-

[1] Quantitative relationships are ignored here; their influence will be discussed in the technical descriptions that follow.

bodies present to sensitize the bacilli or to form the complex which will bind complement. If to such a mixture we add the indicator, red cells sensitized with hemolysin, hemolysis will result because free complement is present. These facts are diagrammatically represented as follows:


A
B. typhosus + antityphoid immune body. + Complement
Complement bound in A, not free to act on B.
B
Red cell + antired cell immune body.
(No hemolysis.)
A
B. typhosus + anticoli immune body. + Complement
Complement not bound in A, and free to act on sensitized red cells.
B
Red cell + antired cell immune body.
(Hemolysis.)

In demonstrating this phenomenon B. typhosus, serum and complement are mixed and incubated for a time necessary for combination to occur, should antibody be present. The red cells washed free of all serum elements are sensitized by adding homologous antiserum, and this mixture is also incubated for a time necessary to insure binding. The latter mixture is added to the first after the appropriate time.

In this discussion we have used B. typhosus as an example. If reference is made to the constituents of bacteria in relation to antibody combination (see under Agglutination), it will be obvious that extracts of bacteria will find immune bodies and, therefore, complement, and that such extracts may be used in place of whole bacteria. The specificity of the fixation will depend on the same factors, specific soluble substance (carbohydrate) and nucleoproteins, as previously described. As has been noted in previous chapters, native albumins are antigenic. They, when sensitized with their specific antibodies, will also bind complement. Neisser and Sachs recommend this procedure for forensic blood tests in place of the precipitin test already described.

In the phenomenon, as described, the suspension of typhoid bacilli would be termed the antigen. If we could substitute the "soluble specific substance," the term antigen would no longer be etymologically correct. As has been emphasized in previous chapters, the term antigen refers to substances which are capable of stimulating the production of antibodies; however, antibody may be specifically bound by non-antigenic substances, which substances may be more correctly termed reagents. This is reiterated at this place because the term antigen is used in a confusing manner most frequently in descriptions of the complement-fixation phenomenon.

Application of the Bordet-Gengou Phenomenon.—Four applications of this reaction should be apparent and theoretically possible: (1) The use of known antibodies to identify or classify unknown microörganisms;

this application has been used to a considerable extent, but has many drawbacks as compared with the simpler agglutination reaction. When satisfactory agglutination antigens are not possible the reaction has considerable value. Closely allied types tend to give greater group reactions than are encountered with the agglutination reaction. These may be eliminated to some extent by careful titration of the reagents. (2) The second application is the demonstration of the presence and the identification of the antibodies in the patient's serum. This application is, therefore, a diagnostic procedure. This procedure is also employed in strengthening the evidence that a microörganism isolated from a disease is the causative agent. Naturally the presence of antibodies does not prove that it is the primary etiological agent of the disease or infection, as the antibodies might develop if the organism were a secondary infecting agent. (3) The third application is the use of the reaction to determine quantitatively the content of complement-fixing antibodies in antibacterial serums to be used for therapeutic purposes. (4) The application of this phenomenon to identify proteins in place of the precipitin reaction. The use of the complement-fixation phenomenon is advocated as being more delicate and applicable where the material available is too little for precipitin tests. As the reaction has had its widest application as a diagnostic procedure, this will be taken up first.

The first practical application to the diagnosis of disease was attempted by Wassermann and Brück[1] with the serum of tuberculous individuals. Their results were interesting but not of practical value. Their later results with syphilitic serum were more encouraging. Testing the serum of monkeys which had been inoculated with human syphilitic material, with an extract of human syphilitic organs, fixation of complement was obtained. A short time later Wassermann, Neisser, Brück and Schucht and others obtained similar results with human syphilitic serum. For this reason complement fixation as applied to the diagnosis of syphilis has been known as the Wassermann reaction. The original technic of Wassermann was based on the presumption that a specific syphilitic substance was present in the organ extracts. With the isolation of the Treponema pallidum specific antigens became available, and it will suffice here to say that the Wassermann reaction, although disease-specific, is not immunologically-specific as in the examples given above. This will be discussed later.

Before considering the application of the complement-fixation reaction the reagents and their utilization will be described:

PREPARATION AND STANDARDIZATION OF REAGENTS.

The reagents used in complement fixation are:

1. Salt solution.
2. Patient's serum, or immune serum.
3. Erythrocytes.
4. Hemolysin.
5. Complement.
6. Antigen or fixation reagent.

[1] Deutsch. med. Wchnschr., 1906, **32**, 449.

The *reaction* of the *reagents* and *glassware* has been found to have an important bearing on the accuracy of the test. *Acidity* and *alkalinity* may give rise to false reactions, which may be positive or negative according to the degree of acidity or alkalinity. Alkali-free glassware should be used. If forced to use lime-glass, soaking in 1 per cent. hydrochloric acid is recommended and thorough rinsing should precede use. This is only a temporary expedient as soluble alkali will be present as long as there is glass.

Salt solution (physiological) should be prepared with distilled water and chemically pure sodium chloride. It should be neutral in reaction to phenolphthalein. As a check, the solution can be titrated volumetrically to determine the percentage of chloride.

The Serum or Transudate for Test.—Human serum is obtained by puncture of the median basilic vein. Animals are bled from a vein, the carotid artery or from the heart. The blood should be allowed to clot, without disturbance. The serum is drawn off after clotting is complete and centrifugalized to remove red cells. Every precaution should be taken to avoid hemolysis as the products of hemolysis may interfere with the test (Anticomplementary action[1]). Contamination and subsequent growth of bacteria in the serum will interfere with the test. All serums should be inactivated before the test even though the serum has been aged for a time sufficient for all complement to deteriorate. The reason is that heating destroys certain interfering substances (anticomplementary) which may be present in stored as well as fresh serums. Stored serum should be kept at low temperature, as near 0° C. as possible. Preservatives should not be added as they may interfere with the test.

Spinal fluid is centrifugalized to throw down cell fibrin etc., and used without inactivation.

Preparation of Erythrocytes.—Red cells from sheep are usually employed. Those from man, ox, or other animals may be used. The blood from sheep is obtained from the jugular vein by trocar, the blood collected in a bottle containing glass beads. Before coagulation can occur, the bottle is shaken thoroughly to defibrinate the blood. The defibrinated blood is filtered through two layers of cheesecloth and the cells washed with saline to free them from serum.

The erythrocytes are washed and prepared in the following manner: 2 to 4 c.c. of defibrinated blood are placed in a 15 c.c. graduated centrifuge tube and the tube is then filled with physiological salt solution. The defibrinated blood and salt solution are mixed by alternately drawing them into a pipette and expelling by means of a rubber bulb. The volume of salt solution should be at least three times that of the defibrinated blood, since otherwise the serum will not be completely removed. The mixture is centrifugalized rapidly for a sufficient time to throw the erythrocytes to the bottom of the tube. The supernatant fluid is decanted, or drawn off with the pipette, and replaced with fresh salt solution. The sedimented eryth ocytes and saline are again mixed and centrifugalized. The process is repeated until the erythrocytes are entirely free from serum.[2] In many laboratories three washings are considered sufficient, but as we have found that even after four washings the serum is not always completely removed, we believe that better results will be obtained by *washing six times*. After the final washing the level of the erythrocytes is marked on the tube with a wax pencil before the cells have been disturbed by the removal of the supernatant fluid. The supernatant fluid is then discarded and the packed erythrocytes are diluted with salt solution to twenty times their volume. This gives us the 5 *per cent. suspension of erythrocytes*. The amount of defibrinated blood per centrifuge tube and the speed and duration of centrifugalization should always be the same, at least for the final washing, in order that the packing of the cells be uniform day by day.

Blood that has been withdrawn from the animal for more than two days in summer or three days in winter is usually unsuitable for hemolytic work, as the

[1] See later.

[2] The presence of serum may be determined by the nitric acid test.

resistance of the erythrocytes becomes weaker and hemolysis occurs too rapidly, so that perfect balance of the hemolytic system cannot be obtained.

In places where it is inconvenient to obtain sheep cells, human erythrocytes have been used. An advantage in the human hemolytic system arises from the fact that by its use a possible error in the test is avoided: human serum frequently contains natural sheep hemolysin and the excess of hemolysin introduced into the hemolytic system when such a serum is being tested may, according to some investigators, cause a weakening or loss of a positive reaction. In our experience this error is eliminated in the Wassermann reaction by the practice of reading the reaction as soon as the controls of the test are completely hemolyzed (see section on Reading and Interpreting the Test, pp. 257 and 258). In tests with bacterial fixing reagents where the fixation of complement is less firm than in the Wassermann reaction the native antisheep hemolysin in human serum may be a source of error.

Preparation of Hemolytic Serum, Hemolysin.—Rabbits are injected intravenously with washed red cells. A 50 per cent. suspension is used. Begin with 0.25 c.c. and increase each succeeding dose by 0.25 c.c. until 2 c.c. is reached. Injections are done every two days. Three days to five days after the last injection theanimal is bled out after an anesthetic is given.

The bleeding is done directly from the heart. A fairly large-sized needle is attached by means of a rubber tube to a pint Blake bottle which has a two-hole stopper. The stopper has two glass tubes. One of the glass tubes is attached to a suction pump, and the other to the needle. The animal is anesthetized to such an extent that the heart is actively beating. In this way practically all the blood can be obtained without any loss through clotting.

When the blood has coagulated, the serum is drawn off, centrifugalized and inactivated at 56° C. for thirty minutes. Then it is mixed with an equal amount of pure, sterile, neutral glycerin,[1] and stored in small vials.

Standardization of Hemolysin.—Hemolysin is standardized for the purpose of determining its hemolytic unit. This is defined as the smallest amount of hemolysin which will give complete hemolysis of 0.1 c.c. of a 5 per cent. suspension of erythrocytes with two hemolytic units of complement, at the end of thirty minutes in the water-bath at 37° C. The standardization may be obtained by titration of one or more dilutions of the hemolysin, and for convenience, the standard dilution of the hemolysin is chosen to be that dilution of the hemolysin in which 0.05 c.c. contains one hemolytic unit. The standard dose of hemolysin for sensitizing erythrocytes is 0.1 c.c. of the standard dilution, or two hemolytic units. The following table shows the scheme of titrating the hemolysin:

HEMOLYSIN TITRATION.[2]

Number of tube.	Hemolytic serum in dilutions to be tested for the unit, c.c.	Two-unit complement.	Five per cent. erythrocyte suspension, c.c.	0.85 per cent. salt solution, c.c.	
1	.1	.1	.1	.1	Incubated for 30 minutes in the water-bath at 37° C.
2	.09	.1	.1	.21	
3	.08	.1	.1	.22	
4	.07	.1	.1	.23	
5	.06	.1	.1	.24	
6	.05	.1	.1	.25	
7	.04	.1	.1	.26	
8	.03	.1	.1	.27	
9	.02	.1	.1	.28	
10	.01	.1	.1	.29	
11	.1	.0	.1	.3	
12	.0	.1	.1	.3	
13	.0	.0	.1	.4	

[1] Longworthy, V., and Wemple, M.: Jour. Lab. and Clin. Med., 1924–1925, **10**, 864.

[2] In the table above, the actual hemolysin titration is comprised in the first ten tubes. Tube 11 is a control of the hemolytic serum for lytic action without the aid of complement. Tube 12 is a control of the complement for lytic action without the aid of hemolysin. Tube 13 is a control of the stability of the erythrocyte suspension.

Sensitizing the Erythrocyte Suspension.—After hemolysin has been standardized it is usually customary to sensitize the erythrocytes for use in tests or for the standardization of other reagents. This may be done by adding two units of hemolysin to each 0.1 c.c. of 5 per cent. erythrocyte suspension. As will be seen later (standardization of the hemolytic system) a slight deficiency of complement may be replaced by increasing quantitatively the amount of hemolysin. During the "fixation period" in the actual test, complement, because of its nature, is deteriorating, and the rapidity of deterioration varies with the temperature. To ensure that the hemolytic system shall be sufficiently active at the end of a (negative) test, this deterioration of complement is provided for by sensitizing the unit volume of the erythrocyte suspension with two units of hemolysin. The danger of obtaining false positive reactions because of an inactive hemolytic system is thus avoided.

The standard technic for sensitizing the cells is as follows: To 1 part of 5 per cent. suspension of erythrocytes add 1 part of the standard dilution of hemolysin. 0.2 c.c. of this mixture will contain 0.1 c.c. of 5 per cent. suspension of cells and two hemolytic units of hemolysin.

The combination of hemolysin, complement and cells is known as *the hemolytic system*. The daily titration of hemolysin or of complement is spoken of as "standardizing the hemolytic system."

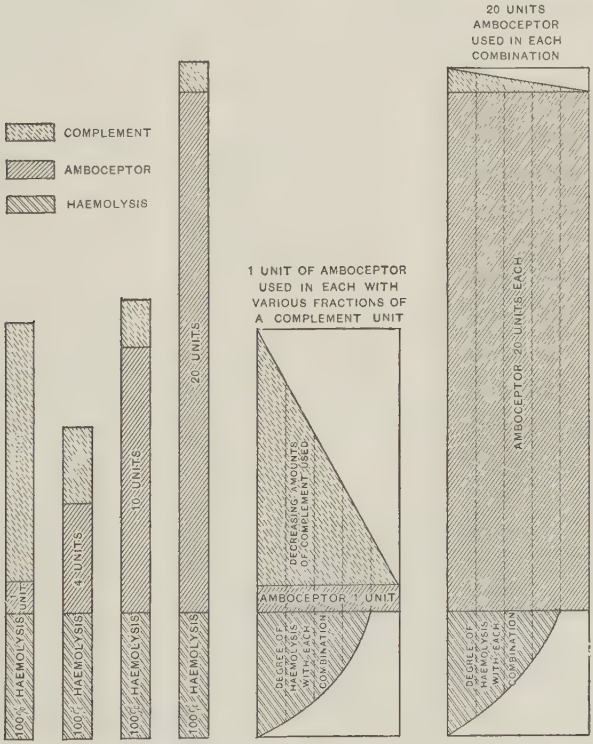

20 UNITS AMBOCEPTOR USED IN EACH COMBINATION
COMPLEMENT
AMBOCEPTOR
HAEMOLYSIS
1 UNIT OF AMBOCEPTOR USED IN EACH WITH VARIOUS FRACTIONS OF A COMPLEMENT UNIT
100% HAEMOLYSIS 1 UNIT
100% HAEMOLYSIS 4 UNITS
100% HAEMOLYSIS 10 UNITS
100% HAEMOLYSIS 20 UNITS
DECREASING AMOUNTS OF COMPLEMENT USED
AMBOCEPTOR 1 UNIT
DEGREE OF HAEMOLYSIS WITH EACH COMBINATION
AMBOCEPTOR 20 UNITS EACH
DEGREE OF HAEMOLYSIS WITH EACH COMBINATION

Fig. 89.—Showing limits of compensation. (Noguchi.)

Standardization of the Hemolytic System.—Complement fixation is, within limits, a quantitative reaction; that is, a sensitized antigen can fix only a limited amount of complement. Moreover, the extent or degree of sensitization of the fixing reagent determines the amount of complement which it can fix. Thus

in an actual complement-fixation test, two or more different amounts of the patient's serum are used with a constant amount of fixing reagent, and the degree of fixation (*i. e.*, the amount of complement fixed) will vary with the different amounts of the patient's serum, the larger amounts of serum leading to the fixation of more complement (in case a positive serum was used). An excess or a deficiency of complement in the test may therefore give rise to errors in diagnosis (since as stated above the amount of complement fixed depends on the degree of sensitization of the fixing reagent). This is perhaps even more evident if we stop to consider the use in the test of the hemolytic system as an indicator of "free" complement. In the hemolytic system it can be shown that a deficiency of either complement or hemolysin may be replaced to a certain extent by increasing quantitatively the other; for example, if one unit of hemolysin and a certain minimum amount of complement acting together will cause complete lysis of a standard quantity of erythrocytes, then one-tenth the amount of complement may accomplish the same result, provided 20 units of hemolysin are used. The same would be true, within certain limits, of the compensating power of complement when the amount of hemolysin is decreased.

The diagram on page 237 will illustrate both the compensation or interchangability of complement and hemolysin, also their limitations.

Thus we see that for accurate results a careful determination should be made of the proper amount of complement to be used in the test; and also that an exact standardization of the hemolytic system is necessary. By such a standardization we mean the method by which the relative amounts of complement and hemolysin are determined, which together will hemolyze 0.1 c.c. of 5 per cent. erythrocyte suspension within a standard time and at a standard temperature.

Preparation of Complement.—Not all fresh serum has the power of reactivating the serum of an alien species or of being "fixed" by an antigen-amboceptor complex, but that of the guinea-pig has unusual power in reactivating human and rabbit sera; hence guinea-pig serum is generally used for complement in complement-fixation tests and in hemolytic work. There is wide variation in the activating power of the serum of individual guinea-pigs, and also in its "fixability" (*i. e.*, its power of combining with fixing reagent and immune serum in the complement-fixation reaction). It is, therefore, advisable, if each pig's serum cannot be tested, to use the pooled serum of at least three guinea-pigs in order that the complement may have average activating and combining properties. To be sure, however, of obtaining efficient complement for complement-fixation tests with bacterial fixing reagents, it is essential that each pig's serum be tested for both activating power and fixability; all complements found by preliminary tests to be suitable are then pooled and titrated to determine the exact dose for use in tests.

Guinea-pigs that have been used for antitoxin tests and other purposes may be of use in complement-fixation work after a rest of three months, but their serum is less liable than that of unused pigs to be of normal activating power. In the complement-fixation test for glanders, pigs that have been inoculated with horse serum should never be used, as substances are formed in their serum that cause a reaction with the serum of the horse that is being tested for glanders. In such an instance the serum controls of the test may in themselves inhibit hemolysis and no reading of the test can be made. Gravid pigs should not be used for complement, as their serum is liable to be weak in activating power.

To obtain complement, guinea-pigs may be bled from the heart by aspiration and used again after several weeks' rest, or they may be bled to death. In our laboratory the pigs after being stunned by a blow at the base of the skull are bled from the throat[1] into Petri dishes, or they may be bled to death from the heart after being chloroformed. In the latter case an incision is made into the

[1] Care must be taken not to cut the esophagus, as the stomach contents may thereby be mixed with the serum. If this occurs, the serum is unfit for use in complement-fixation work.

thoracic cavity; the heart is grasped with forceps and cut into with sterile scissors. The blood is collected in sterile, neutral Petri dishes, left at room temperature protected from light for thirty minutes, then placed in the ice-box at 0° C. to 6° C. overnight. The serum is drawn off with a capillary pipette and centrifugalized to free it from blood cells. Before being pooled the serum from each pig should be tested separately for natural hemolysin, which is occasionally present, and for activating power. Serum containing natural hemolysin, or showing weak activating power should be discarded.

Complement deteriorates rapidly if exposed to sunlight or to a warm temperature (over 20° C.); if kept in the ice-box (at a temperature below 10° C.) it remains potent for at least forty-eight hours. Complement may be preserved for several weeks by freezing. Its activating power is thus retained, but its capacity for being bound may weaken so that it may not be reliable for use. In our experience complement frozen for one week is still perfectly satisfactory.

Complement is used in a 10 per cent. dilution made with physiological salt solution.

Standardization of Complement.—The standardization methods vary slightly according to the purpose of the complement-fixation reaction. The following gives the general procedure in the diagnostic test. Variations from this procedure will be noted in the later descriptions of special procedures.

The guinea-pig serums are kept separate until standardization tests are made. First test for the presence of natural sheep hemolysin and the hemolytic capacity of the complement according to the following scheme.

PRELIMINARY TESTS OF INDIVIDUAL GUINEA-PIG SERUMS.

Test No.	Purpose of test.	Guinea-pig serum, 1 to 10 dilution, c.c.	0.9 per cent. saline, c.c.	5 per cent. suspension c.c.	Sensitized cells.
1	Test for natural antisheep amboceptor	.2	.2	.1	0
2	Test for hemolytic activity	.1	.2	0	.2

Place the tests in the water-bath at 37° C. and at the end of thirty minutes read the reactions in each tube.

If Test No. 1 shows no hemolysis, natural hemolysin is absent. If Test No. 2 shows complete hemolysis, the complement is sufficiently active. The complement serums may then be pooled and stored undiluted until used.

Titration of Pooled Complement for Diagnostic Test.—Two hemolytic units of the complement are used. By hemolytic unit of complement is meant the smallest volume of complement that at the end of thirty minutes in the water-bath at 37° C. will completely hemolyze 0.1 c.c. of sheep's erythrocytes that have been sensitized with two standard units of antisheep amboceptor. The pooled complement must be titrated every day with fresh sensitized cells. In this method for

balancing the hemolytic system by a complement titration, instead of by a hemolysin titration the hemolysin is used in its standard dilution every day. The new hemolysin is standardized by the method described (see page 236) and ever after is used in its standard dilution. The reason for this is that the titer of the hemolysin does not change, while every new complement is an unknown quantity both in regard to hemolytic activity and fixability until it has been tested. Therefore, in order that the various complements used for testing patients' serums from week to week may have as nearly as possible the same hemolytic and fixation value, we titrate the variable (the complement) against the constant (the hemolysin). As the new hemolysin was standardized with 2 hemolytic units of a known complement, the balance of the system is kept as nearly the same as possible for each days' tests.

Method for Daily Titration of Complement for Hemolysis and Fixation.—*How to Determine the Unit of Hemolysis.*—By hemolytic unit of complement is meant: the smallest amount of complement which will completely hemolyze 0.1 c.c. of a 5 per cent. suspension of erythrocytes which have been sensitized with 2 hemolytic units of hemolysin, at the end of thirty minutes in the water-bath at 37° C.

Dilute the pooled complement 10 per cent. (1 part complement plus 9 parts saline) and titrate that dilution with 0.2 c.c. of sensitized cells, and saline solution to bring the total volume to 0.5 c.c. Place this titration in the water-bath at 37° C. and at the end of thirty minutes read the unit of hemolysis.

The reagents for the complement titration are pipetted in accordance with the following scheme:

COMPLEMENT TITRATION.

Tube No.	Complement, 1 to 10 dilution, c.c.	Saline, c.c.	Sensitized cells, c.c.	Five per cent. suspension of erythrocytes.
1	.1	.2	.2	0
2	.09	.21	.2	0
3	.08	.22	.2	0
4	.07	.23	.2	0
5	.06	.24	.2	0
6	.05	.25	.2	0
7	.04	.26	.2	0
8	.03	.27	.2	0
9	.02	.28	.2	0
10	.01	.29	.2	0
11	0	.3	.2	0
12	.2	.2	0	.1

If Tubes 1, 2, 3, 4, 5, 6, 7 and 8 show complete hemolysis, and Tubes 9 and 10 show partial or no hemolysis, the amount of complement in Tube 8 represents the hemolytic unit of complement.

Tube 8 contains 0.03 c.c. of the complement dilution. For diagnostic tests we use 2 hemolytic units, or 0.06 c.c., of the complement dilution. For convenience of pipetting, we make a fresh dilution of complement, of which 0.1 c.c. will contain 0.06 c.c. A simple method for making the new dilution is as follows: To 6 parts of 10 per cent. complement add 4 parts of saline. This enables us to pipette the 0.06 c.c. in 0.1 c.c. of the new dilution.[1]

[1] It must be remembered that the unit of complement, although expressed as 0.03 c.c., does not lie exactly in the volume indicated, but lies between that and the next volume. This fact has to be considered when very fine titrations are desired.

Interpretation of the Complement Titration.—Tubes 1 to 10 are tests of the hemolytic activity of the complement. Tube 11 is a control of the stability of the sensitized cells. If any degree of hemolysis occurs in Tube 11, the cells are unstable and not suitable for complement-fixation tests. Tube 12 is the same as Test 1 in the preliminary tests. If any degree of hemolysis occurs in Tube 12, it invalidates the hemolysis in Tubes 1 to 10.

PRELIMINARY TESTS OF INDIVIDUAL GUINEA-PIG SERUMS FOR FIXATION.

Test No.	Purpose of test.	Fixing reagent standard dilution, c.c.	Serum, c.c.	Complement 2-units dilution, c.c.	0.9 per cent. saline, c.c.		Sensitized cells, c.c.
	Anticomplementary with antigen	.4	0	.1	..	After fixation period, add sensitized cells	.2
1		.3	0	.1	..		.2
		.2	0	.1	..		.2
		.1	0	.1	.1		.2
2	Anticomplementary with serum, a known positive serum, undiluted	0	.04	.1	.2		.2
3	Fixability	.1	.01	.1	.1		.2
Controls:							
4	Complement stability	0	0	.1	.2		.2
5	Sensitized cell stability	0	0	0	.3		.2

Standardization of Complement for the Qualitative Diagnostic Test with Bacterial Fixing Reagent.—The earlier writers recommended the use of a pool of several guinea-pig serums in order to avoid variation in the complements used from day to day, although they were all agreed that there was no specificity of complement. In our titration of immune serums we found that there is considerable specificity for fixability among guinea-pig serums, as well as the variation in hemolytic activity and anticomplementary properties recognized by all workers. The latter variants can be controlled by dilution of the complement, but if the serum is lacking in fixability it should be excluded from the pool, as it may weaken, or render negative, the value of the whole pool. The hemolytic and fixation strengths of a serum are not always proportionate, that is, one serum may have a low hemolytic titer and give a weak or negative fixation reaction. On the other hand, a serum of high hemolytic activity will often be very strongly fixed by the fixing-reagent-antibody combination. This type of serum has given us some of our highest titers of immune serums. To obtain an efficient complement for bacterial fixation, it is essential, we repeat, that every pig's serum be tested for fixation as well as for hemolysis before pooling. This preliminary test establishes the value of the complement from each pig, and does away with the necessity for pooling in order to avoid variations. This is of practical value to the small laboratory where the number of tests does not require a large volume of complement. The good complement from one pig is

just as efficient as the good complement from ten pigs, and much better than a pool of good and bad serums.

Method for Making Preliminary Tests for Fixation before Pooling the Complement.—These tests are used for microbal fixing reagents (antigens) as well as for lipoidal extracts. For this purpose we make a complete titration of the 10 per cent. dilution of each pig serum. It must be kept in mind that our preliminary tests are for individual serums, not for pooled complement. It is essential to know whether the individual pig complements will be anticomplementary with the bacterial fixing reagent, or with the control positive serum, and whether the individual pig complements will be fixed by the fixing-reagent-serum complex. The tests on page 241 determine those points:

Place these tests in water-bath at 37° C. for one hour. Then add 0.2 c.c. of sensitized cells to each tube, and replace the tests in the water-bath for thirty minutes. Then read the reaction in each tube.

The reactions in preliminary tests are interpreted as follows:

Interpretation of the Preliminary Tests.

Test No. 1. Must be completely hemolyzed in 0.1, 0.2, 0.3 and have only a trace of fixation in 0.4.

Test No. 2. Must be completely hemolyzed.

Test No. 3. Must be completely fixed. If in 10 pigs tested only 1 or 2 show a trace of hemolysis in the No. 3 test, they can be included in the pool, but any pig's serum giving only weak or no fixation should be excluded from the pool.

Test No. 4. Must be completely hemolyzed in eight to ten minutes.

Test No. 5. Must show no trace of hemolysis.

To Find the Exact Dose of Pooled Complement for Tests.—All serums which by the preliminary tests are found suitable for the desired complement fixation, are pooled, and the pooled complement is titrated by the method described on page 239.

The methods for standardizing complement for quantitative diagnostic tests and for standardization of immune serums are given in the paragraphs describing those tests. Pages 248 and 245.

Preparation of Specific Fixing Reagents (Antigens).—The method of preparing microbral fixing reagents varies considerably with the particular organism on hand. The points that must be considered are: (1) the optimum medium and period of incubation for the growth of the organism; (2) the method of treating the microbral growth in order to obtain a fixing reagent that answers the requirements for specific complement fixation. It is therefore necessary to describe the preparation of the individual fixing reagents.

B. Tuberculosis.—1. *Defatted Dried Bacillary Fixing Reagent.*—The bacilli are grown in glycerin-broth[1] for three months. The culture is then killed by heating in the Arnold sterilizer for one hour. The culture is filtered through filter paper. The filtrate is discarded, and the residue treated as follows: It is first washed in sterile water to remove all traces of broth. Then it is placed in absolute alcohol in the proportion of 1 volume of residue to 10 volumes of alcohol. This mixture is placed in the ice-box for two weeks. It is then centrifugalized for ten minutes and the supernatant fluid discarded. To the sediment is added 10 volumes of fresh absolute alcohol. This mixture is placed in the water-bath at 56° C. for one hour. It is then centrifugalized for ten minutes, and the supernatant fluid is discarded. To the residue add 10 volumes of ether. This mixture is stirred constantly at room temperature for one hour, centrifugalized ten minutes, and the supernatant fluid is discarded. The centrifuge tube containing the residue is plugged with sterile cotton and placed in the dark at room temperature for twenty-four to

[1] See Medium for Tuberculin.

forty-eight hours, until the residue is perfectly dry. This is usually accomplished in twenty-four hours. The dried powder is emulsified in a large mortar by trituration with 0.9 per cent. saline solution, in the proportion of 1 gram of powder to 200 c.c. of saline. The emulsion is heated for one hour at 80° C. The antigen is now ready for use, and is standardized as described on page 244.

Moist-Bacillary Fixing Reagent.—The bacilli are grown as described above. The broth culture is placed in the Arnold sterilizer for one hour. It is then filtered through folded paper and the filtrate is discarded. The residue is washed in sterile 0.9 per cent. saline solution to remove all traces of the broth. It is then centrifugalized for ten minutes and the supernatant salt discarded. The moist residue is emulsified by trituration with saline in a large mortar. This emulsion is heated for one hour in water-bath at 80° C. It is now ready for use, and is standardized by the method described on page 244.

Gonococcus.—A polyvalent fixing reagent is made of five strains which were isolated and identified by Torrey as representing most of the known serological types of gonococcus.

The cultures are grown on North medium for thirty-six to forty-eight hours. The growth is scraped into absolute alcohol. This mixture is placed in the water-bath at 56° C. for thirty minutes, stirring constantly with a glass rod. If the supernatant alcohol has any trace of color, the alcoholic washings must be repeated until the alcohol is colorless. It is then centrifugalized for ten minutes and the supernatant alcohol is discarded. To the residue add 10 volumes of ether and stir constantly at room temperature for thirty minutes. Then centrifugalize for ten minutes, and discard the supernatant ether. The centrifuge tube containing the residue is now plugged with sterile cotton and is placed in the dark at room temperature until the residue is perfectly dry. This is usually accomplished in twenty-four hours, but it sometimes takes forty-eight hours to complete drying. The bacterial suspension is made by triturating the dried gonococci with 0.9 per cent. saline in a large mortar, in the proportion of 1 gram of the powder to 200 c.c. of saline. The bacterial suspension is placed in a sterile bottle, plugged, and heated at 80°C. for one hour. It is now ready for standardization.

Meningococcus.—A polyvalent fixing reagent of the Gordon types is usually employed.

The meningococci are grown on medium "D" for twenty-four hours. The growth is scraped into absolute alcohol and, after thorough shaking by hand, is placed in the ice-box for two days. The bacteria are then precipitated by centrifugalizing and the sediment is washed twice in absolute alcohol. After the second washing the supernatant alcohol is discarded and about 1 c.c. of ether is added to the sediment and thoroughly mixed with it. It is left at room temperature over night. The dry sediment is then thoroughly ground in a mortar and a concentrated suspension of the ground powder is made in physiological saline. This is heated at 65° C. for one hour.

Glanders.—A fixing reagent from a number of strains is made.

Pf. Mallei.—The bacilli are grown on glycerin-potato agar for twenty-four hours. The method for making the fixing reagent is the same as for the gonococcus.

Pertussis Bacillus.—A fixing reagent is prepared of the two serologically different types—A and B.

The cultures are grown on chocolate agar for twenty-four hours. The growth is scraped into 1 per cent. formalin in physiological saline, thoroughly shaken by hand to break up clumps and placed in the ice-box for twenty-four hours. It is again shaken thoroughly and filtered through cotton to remove clumps and centrifugalized at high speed. The sediment is washed twice in 0.05 per cent. formalin in physiological saline, and after the second washing the sediment is suspended in 0.05 per cent. formalinized saline, and is shaken in a mechanical shaker for seven hours.

We have not as yet tried the preparation of this fixing reagent by the method described for meningococci. In all probability that method would give even more satisfactory results.

Influenza Bacillus.—A mixed fixing reagent is made from twelve or more strains. The influenza bacilli are grown on coagulated horse-blood agar for forty-eight hours. The fixing-reagent plates are made from same medium. The growth on plates is treated by the method described for gonococcus fixing reagent.

Typhosus Bacillus.—Prepared in same manner as meningococcus fixing reagent or the gonococcus.

Streptococcus.—Prepared in same manner as meningococcus fixing reagent.

Standardization of Fixing Reagent.—There are two requirements for a good fixing reagent, (1) a long range[1] and (2) specificity. The range of a fixing reagent is determined by mixing varying amounts of the fixing reagent with a constant amount of a previously tested homologous immune serum, a constant amount of complement and a constant amount of sensitized erythrocyte suspension. The specificity of a fixing reagent is determined by using heterologous instead of homologous immune serum in the titration. The methods employed in our laboratory in the titration of fixing reagents are given below.

One or more arbitrary dilutions are made of the fixing reagent using physiological saline as a diluent. Then a titration is made of each dilution according to the diagram given below.

FIXING REAGENT TITRATION.

Number of tube.	Immune serum, c.c.	Fixing reagent dilution, c.c.	Two unit complement, c.c.	0.85 per cent. saline, c.c.		Sensitized erythrocyte suspension, c.c.	
1	0.01	0.25	0.1	0.0	Incubation period. See pages 247 and 248.	0.2	Incubated in the water-bath at 37° C. until all the hemolytic controls are hemolized.
2	0.01	0.2	0.1	0.0		0.2	
3	0.01	0.15	0.1	0.05		0.2	
4	0.01	0.1	0.1	0.1		0.2	
5	0.01	0.05	0.1	0.15		0.2	
6	0.01	0.025	0.1	0.2		0.2	
7	0.0	0.4	0.1	0.0		0.2	
8	0.0	0.3	0.1	0.0		0.2	
9	0.0	0.2	0.1	0.05		0.2	
10	0.0	0.1	0.1	0.1		0.2	
11	0.0	0.05	0.1	0.15		0.2	
12	0.02	0.0	0.1	0.2		0.2	

If fixation is complete through 0.025 c.c. (tube 6), a higher dilution of the fixing reagent should be made, and titrated in the same manner to determine the unit of fixation. *The unit* is the smallest amount of fixing reagent that, with 2 units of homologous immune serum (or 0.01 c.c. of a human serum which has given a + + + + reaction, according to our scheme) gives complete fixation of complement. *The anticomplementary dose* is the smallest amount of fixing reagent that is in itself inhibitory of hemolysis. The longer the range of the fixing reagent the greater is the probability of success in diagnosis. An amount of fixing reagent that fixes complement completely with a serum of high antibody content may give incomplete or no fixation with a serum of low antibody content. Hence in making diagnostic tests, where the detection of even a small amount of antibody is desired, it is advisable to use much more than 1 unit of fixing reagent. As the maximum amount of fixing reagent that may be used with safety is one-fourth the anticomplementary dose, a fixing reagent of long range is necessary. If in the tables above inhibition is complete in the first 5 or 6 tubes, and if hemolysis is complete in tubes 8 and 12, and there is very slight inhibition

[1] The range of a fixing reagent is the difference between the anticomplementary dose (the smallest amount of fixing reagent that is in itself inhibitory) and the minimum fixing dose, the fixing reagent unit.

in tube 7 only, then 0.1 c.c. is the amount of fixing reagent in the given dilution to be used in diagnostic tests. If one-fourth the anticomplementary dose gives complete fixation with a heterologous immune serum, the fixing reagent is nonspecific and unsuitable for tests. Occasionally a fixing reagent is lytic for erythrocytes. In such a case tubes containing the largest amount of fixing reagent show more hemolysis than those containing less. The fixation curve instead of dropping (Fig. 90, *a*) first rises and then drops again (Fig. 90, *b*). If a lytic fixing reagent is also anticomplementary and has a long fixation range it may be used, otherwise it should be discarded.

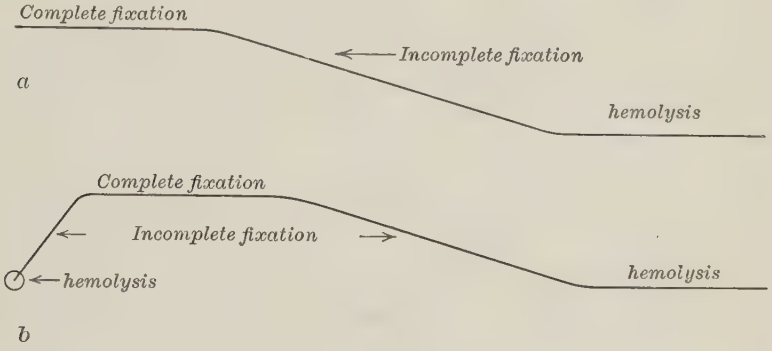


FIG. 90

The Application of the Complement-fixation Reaction to the Titration of the Antibody Content of Serums.—An immune serum may be standardized by means of an antibody content titration. This is made for the purpose of measuring as accurately as possible the antibody unit of the serum. An *antibody unit* is the smallest amount of serum that with the 2 units of a homologous fixing reagent gives complete fixation of complement under standard conditions. The number of antibody units per cubic centimeter may be calculated by dividing 1 c.c. by the minimum fixing dose; that is, if 0.05 c.c. of a 1 in 10 dilution is the antibody unit, the number of antibody units per cubic centimeter is $\frac{1}{0.005} = 200$. An antibody content titration is used for the standardization of antimicrobic sera, such as antigonococcus and antimeningococcus horse sera.

The tests upon which the standardization of a new serum is based consist of titrations of the new bleeding in comparison with the standard titer of a known serum. In order that the comparison may be true, it is necessary that the reagents used in making the tests shall be as nearly as possible constant in their reactions, leaving as the only variable the unknown serum to be tested. This would be a simple matter were it not for the fact that the complement is also a variable factor, and attempts to render it constant are not always successful. It is true, then, in this as in all other complement-fixation work, that the value of the tests depends upon the accurate measurement of the dose of comple-

ment. Formerly[1] [2] this complement dose was measured in regard to its hemolytic strength, an exact hemolytic unit being used with two units of hemolysin. If the fixation and hemolytic units of a given complement were always proportionate this method for determining the complement dose would be an advantage, as a clear-cut hemolytic reaction is quickly and easily obtained when the complement is in good condition. On the other hand[3] the hemolytic and fixation strengths of guinea-pig serums are many times diverse, and to measure the dose of complement by its hemolytic strength will not give an accurate fixation titer of the new immune serum The method described below is now employed in this laboratory for the standardization of our meningococcus horse serums.

Standardization of Complement for the Quantitative Determination of the Antibody Content in Immune Serums.—*Tests for the Fixation Unit of Complement with a Standard Fixing Reagent (Antigen).*—In these tests a mixed, or when possible a polyvalent, reagent (antigen) is made from the strains from which the immune serum was made. This reagent is always used in its standard dilution.

The complement from each guinea-pig is given the preliminary tests described on pages 239 and 242. After the results of the preliminary tests are obtained, those complements which are found to have adequate fixation and hemolytic strength for meningococcus fixation are pooled and the pooled complement is tested to determine the exact dose to be used. This is accomplished by titrating the standard dilution of a known meningococcus serum with two standard units of meningococcus fixing reagent and with varying dilutions of the pooled complement. For the standard dose of complement to be used in testing the unknown serums we use 0 1 c.c. of that dilution of the pooled complement which gives the standard titer of the known serum in the required time and complete hemolysis of all hemolytic controls within thirty minutes.

Titration of Unknown Serums.—Having found the most favorable dose of complement the unknown serums are titrated with that in the presence of two standard units of the meningococcus fixing reagent. The horse serums are stored without preservative. They are inactivated by heating in the water-bath at 56° C. for thirty minutes. Each bleeding is titrated in 1 to 10 and 1 to 100 dilution. Up to the present time the titers of our horses have been found within that range.

The titrations are placed in the water-bath at 37° C. for thirty minutes for fixation of the complement. At the end of that time the indicator, consisting of 0.1 c.c. of sheep cells sensitized with 2 units of hemolysin, is added and the tests are replaced in the water-bath until the hemolytic controls are hemolyzed. The reactions are read by the reading method employed for diagnostic tests. If desired, the titrations may be placed in the ice-box overnight and the reactions read from the supernatant fluid in the morning.

[1] Olmstead and Luttinger: Complement Fixation in Pertussis, Arch. Int. Med., 1915, **16**, 67.

[2] Hitchens: Jour. Immunol., 1916, **1**, 4.

[3] Wilson: Proc. New York Path. Soc., N. S., Nos. 6–8, vol. **17**.

The reagents and serum are pipetted in accordance with the following scheme:

ANTIBODY CONTENT TITRATION.

Number of tube.	Immune serum diluted 1 : 10 (etc.), c.c.	Fixing reagent in standard dilution, c.c.	Standard complement, c.c.	0.9 per cent. saline, c.c.		Sensitized erythrocyte suspension, c.c.	
1	0.2	0	0.1	0	Fixation time and temperature for meningococcus horse serums is: thirty minutes water-bath at 37° C.	0.2	After adding sensitized cells, place the racks in water-bath at 37° C. until all the hemolytic controls are hemolyzed.
2	0.1	0	0.1	0		0.2	
3	0.2	0.1	0.1	0		0.2	
4	0.1	0.1	0.1	0		0.2	
5	0.08	0.1	0.1	0.02		0.2	
6	0.06	0.1	0.1	0.04		0.2	
7	0.05	0.1	0.1	0.05		0.2	
8	0.04	0.1	0.1	0.06		0.2	
9	0.03	0.1	0.1	0.07		0.2	
10	0.02	0.1	0.1	0.08		0.2	
11	0.01	0.1	0.1	0.09		0.2	
12	0	0.2	0.1	0		0.2	

When the above titration is used for a quantitative diagnostic test, the time and temperature for fixation period are the same as for the qualitative test for the disease in question. See pages 247 and 248.

The Application of the Complement-fixation Reaction in the Diagnosis of Bacterial Infections.—The practical application of this test is still limited to comparatively few diseases, but in these diseases it is a valuable aid to the clinician in confirming his diagnosis.

The Qualitative Complement-fixation Test.—The method of performing the tests and the reading and interpretation of the results is similar to that for the Wassermann reaction (page 254). It should be kept clearly in mind, however, that with the bacterial antigens (fixing reagents) described the fixation is specific for the causative bacterium. In the Wassermann test for syphilis this is not the case. (See later discussion.)

The kind of fixing reagent, fixation period and temperature for various bacterial diseases is as follows.

For Gonococcus Complement Fixation.

Fixing reagent: Saline suspension of defatted, dried gonococci.

Fixation: Water-bath one hour at 37° C.

For Tuberculosis Complement Fixation.

Fixing reagent: Saline suspension of defatted, dried tuberculosis bacilli.

Fixation: Water-bath one hour at 37° C.

For Streptococcus Complement Fixation.

Fixing reagent: Saline suspension of defatted, dried streptococci.

Fixation: Water-bath thirty minutes at 37° C.

For Pertussis Complement Fixation.

Fixing reagent: Saline suspension of formalinized Bordet-Gengou bacilli.

Fixation: Water-bath one hour at 37° C.

For Glanders Complement Fixation.

Fixing reagent: Saline suspension of defatted, dried mallei bacilli.

Fixation: Overnight in ice-box at 8° to 10° C.

For Meningococcus Complement Fixation.

Fixing reagent: Saline suspension of dried powdered meningococci.

Fixation: Water-bath one hour at 37° C.

The Quantitative Complement-fixation Diagnostic Test.—*Reagents:* Same as for the qualitative test.

Purpose of the Quantitative Test.—To determine the exact antibody content of the patient's serum. (See page 245 "The Titration of the Antibody Content of Serums.")

Method: Titration of varying amounts of patient's serum with constant amounts of all reagents.

Operations of the Test.—1. Standardization of the complement; 2, quantitative test of patient's serum.

1. *Standardization of the Complement.*—The complement is selected by preliminary tests 1 to 5, as described on pages 239 and 242. The pooled complement is then diluted 1 to 10, 1 to 15 and 1 to 20. In the quantitative test, the hemolytic titration of the pooled complement is omitted. There are controls of the hemolytic activity of the complement, and our test is particularly concerned with the fixation unit of the complement.

A known positive serum is diluted to its standard dilution, and is inactivated in the water-bath at 56° C. for thirty minutes. This standard serum dilution is titrated for its antibody content with each of the dilutions of complement and two fixation units of the fixing reagent. For this titration, pipette all reagents as shown in the "Antibody Content Titration" table on page 247. Use the fixation time and temperature for the disease in question. For example: If a quantitative test for gonococcus antibody is being made, place the titrations in the water-bath for one-hour at 37° C. If the test is for syphilis reagin with cholesterinized fixing reagent, place the titrations in the water-bath at 37° C. for one hour. If the test is for syphilis reagin with crude alcoholic extract fixing reagent, place the titrations in the ice-box for four hours. These preliminary titrations must be accompanied by the following controls: Tubes 7, 8, 9, 10, 11 and 12 as described in the qualitative test on page 257. At the end of the fixation period add 0.2 c.c. of the sensitized cells (see page 237) to each tube of the titration and controls. Place in the water-bath at 37° C. until the control tubes 7, 8, 9, 10, 11 and 12 have completely hemolyzed. This should be accomplished in fifteen minutes with some one of the titrations, depending upon the dilution of the complement. When the hemolytic controls are hemolyzed, read the unit of fixation of the antibody-content titration. With one of the dilutions of complement the standard unit of the known serum will be obtained.

For the quantitative diagnostic test use that dilution of the complement with which the standard fixation unit of the known serum is obtained. For example: A syphilitic serum, whose fixation unit is 0.002 c.c. of undiluted serum, is diluted 1 to 10. This dilution is titrated with 2 fixation units of lipoidal fixing reagent and with 0.1 c.c. of each of

the complement dilutions. With the 1 to 10 complement the fixation unit of the serum is 0.01 c.c. and the hemolytic controls are all completely hemolyzed. With the 1 to 15 complement the unit of fixation of the serum is 0.002 c.c. and the hemolytic controls are completely hemolyzed. With the 1 to 20 complement the fixation unit of the serum lies between 0.002 and 0.001 and the hemolytic controls are a trifle cloudy. Thus, the standard unit of the known serum is obtained with the 1 to 15 complement, and that complement dilution is used for the quantitative test of the unknown patient's serum. This dose of complement, *i. e.*, 0.1 c.c. of that dilution which in the presence of 2 fixation units of fixing reagent, with the standard fixation time and temperature, will give the standard unit of the known serum, we have termed: *the standard complement unit.*

Our reason for using a standard complement unit for quantitative tests is this: In the case of bacterial immune animal serums, such as antimeningococcic horse serums, or antigonococcic rabbit serums, the antibody is stable, that is, the complement-fixation unit is not diminished by long standing. Our serums are stored without preservative in the ice-box. In the case of human serums the antibody is relatively stable. The fixing reagents are always stable when made and stored by our methods. Therefore, the only variable in the test is the complement. The quantitative test is usually required in cases where the clinician is observing the patient over a long period of time, making bleedings for complement-fixation tests at frequent intervals. In order that the complement-fixation curve of the patient may indicate as truly as possible the antibody content it is essential that the different lots of complement used for tests throughout the course of observation of a patient, have the same value for fixation as well as for hemolysis. We have found our standard complement unit to give the most consistent curves.

2. *The Quantitative Test.*—Having found the standard complement unit, we now make titrations of 1 to 10 and 1 to 100 dilutions of the patient's serum, in accordance with the scheme for "Antibody Content Titration" on page 247. We have found that titrations can be made with less error and more quickly when varying amounts of a single dilution are pipetted with one pipette, as shown in our scheme, than when separate dilutions are made for each tube, as recommended by other workers.

Fixation time and period are the same in the test of patients' serum as in the preliminary titrations for finding the standard complement unit. Controls 7, 8, 9, 10, 11 and 12 must accompany all diagnostic tests.

Readings.—The unit of fixation of each serum dilution is recorded, and the reactions in all control tubes are recorded.

Quantitative Diagnosis.—We report to the clinician the exact complement fixation unit (antibody content) in terms of undiluted serum. For example: If the unit of complement fixation is 0.03 c.c. of the 1 to 10 dilution, we report: "Antibody unit is 0.003 c.c. of undiluted serum." Or, if the unit is 0.04 of the 1 to 100 dilution, we report: "Antibody unit is 0.0004 c.c." In our opinion the exact unit from week to week gives the clinician more information than when the

same reaction is reported as, "Six plus," or, "Twenty plus,"[1] or, as "Very strongly positive."[2]

The Present Status of the Complement-fixation Test in the Diagnosis of Bacterial Disease.—*In the Diagnosis of Gonococcus Infection.*—The Bordet-Gengou[3] reaction was applied to the diagnosis of gonococcus infections by Muller and Oppenheim[4] in 1906, and in the same year Brück[5] working with Wassermann made a comparative study of the fixation and agglutination reactions. Later (1907) Teague and Torrey,[6] and Watabiki,[7] attempted to group all gonococci by the complement-fixation test.

Schwartz and McNeil[8] (1911) made a comprehensive study of the reaction in relation to the clinical diagnosis and drew decisive conclusions favorable to the value of the test. Their technic has been widely followed and many later workers[9 10 11 12 13 14 15 16 17 18] have corroborated their results. Schwartz and McNeil used a polyvalent fixing reagent made from the ten strains identified by Torrey. This was an aqueous extract of living gonococci. Other kinds of fixing reagent have been described by later workers, notably Warden's "specific fats" and Thomson's dissolved whole cocci. Wilson, of this laboratory, working with McNeil, in 1916, developed the defatted fixing reagent described on page 242, and since then this lipoid-free product has been used in many of the New York laboratories, and in several other cities throughout the country. The advantage of the defatted fixing reagent is its stability, freedom from anticomplementary reaction and comparatively high titer. The aqueous products were always used in low dilution and would deteriorate in a few weeks or months, while the heated suspensions of defatted cocci have an average titer of 1 to 20 dilution and are perfectly stable, retaining their antigenicity for more than a year. This is a practical advantage, as it enables one to make up a large stock at one time, thus reducing the cost of production. As regards the value of the defatted fixing reagent in "picking up" more positive reactions in cases having a small amount of the gonococcus antibody, a comparison of our results with the publications of workers using various other kinds of fixing reagent, shows practically the same percentage of positives in the same clinical types of cases, and would indicate that the antigenic property is not confined to any particular part of the coccus body. The reports of studies on gonococcus complement fixation show an agreement of opinion on the following points:

[1] McNeil: Jour. Lab. and Clin. Med., 1921, **7**, 109.
[2] Kolmer: Am. Jour. Syph., 1922, **6**, No. 3.
[3] Ann. de l'Inst. Pasteur, 1907, **15**, 290.
[4] Wiener klin. Wchnschr., 1906, **19**, 894.
[5] Deutsch. med. Wchnschr., 1906, **23**, No. 34, 945; **44**, 10 and 110; **9**, 588.
[6] Jour. Med. Res., n. s., **12**, 236.
[7] Jour. Med. Res., 1909, **20**, 365.
[8] Am. Jour. Med. Sci., **144**, 815.
[9] Swinburne: Arch. Diag., July, 1911.
[10] Keyes: Am. Jour. Med. Sci., January, 1912.
[11] Schmidt: Tr. Am. Urol. Assn., 1911.
[12] Gradwohl: Am. Jour. Derm. and Syph. Urin. Dis., June, 1912.
[13] Kolmer: Text-book of Infection Immunity and Biology Therapy, 3d ed., 1923.
[14] Warden: Jour. Infect. Dis., 1915, **16**, 426.
[15] Thomson: Lancet, July 13, 1918, p. 42.
[16] Smith and Wilson: Jour. Immunol., 1920, **5**, 499.
[17] Torrey and Wilson: Jour. Infec. Dis., 1922, **31**, 148.
[18] Wilson and Forbes: Jour. Immunol., 1923, **8**, 105.

Cases of anterior gonorrheal urethritis and acute vulvo-vaginitis rarely give a positive complement-fixation test.

A positive reaction is indicative of the presence, or recent activity, in the body of a focus of living gonococci.

A negative reaction does not exclude gonococcus infection.

A strong positive reaction is not to be expected before the fourth week. A positive reaction does not disappear entirely until seven or eight weeks after cure.

Persistently negative results obtained through a considerable period of time indicate the probability of a cure.

The gonococcus complement-fixation test is specific as regards human serums. We have made specificity tests upon a large number of human serums and spinal fluids, from cases of syphilis, tuberculosis, meningitis and poliomyelitis, and obtained negative reactions in all, except a few of the syphilitic serums which had a coincident history of gonorrhea.

In the Diagnosis of Tuberculosis.—Complement fixation has been studied in its relation to tuberculosis from several points of view; from the standpoint of its bearing on clinical medicine to discover whether it was of value in the diagnosis, prognosis or in controlling the specific therapy of tuberculosis in man; from the standpoint of pure pathology to determine what light it might throw upon the essential nature of the disease and immunity thereto; and from the position of the veterinarian who is in search of reliable diagnostic methods. The chief investigation has centered around the diagnostic and prognostic possibilities of the reaction. In the interest of the individual patient, as well as of public health, early diagnosis is essential, and the complement-fixation test has been sought as an aid in this direction. The earlier workers[1] on this subject reported such contradictory results that little

[1] The finding of complement fixation in tuberculous patients appears to be dependent upon the interplay of two, and possibly three, fundamental factors: (1) The antigens liberated in the body as a result of the invasion of the tuberculosis bacillus. The exact nature of these is still unknown. They may conceivably consist of the bacillus itself, of products of the disintegration of the bacillus, of endotoxins, or of products of the disintegration of the tissues evolved during the tuberculous process. (2) The antibodies which may be produced in response to the stimulus exerted by the antigens. (3) We must consider the rôle possibly played by the inhibiting substances that are stated by Calmette and Caulfield to occur in certain tuberculous serums, and which have the effect of producing negative reactions in serums which contain antibodies. The interaction of these factors in various quantitative relations may give rise to various hypothetical contingencies in the test reactions of a serum, the most important of which appear to be the following:

Case 1.—No antigen is or has been present in the body, therefore we obtain a negative reaction since there is also no antibody.

Case 2.—Antigen is present but for some reason there is no antibody formation, and the reaction is negative.

Case 3.—Antigen is present, but the antibody is quantitatively insufficient to more than saturate the antigen; it therefore remains undetected, and the reaction is negative.

Case 4.—Antigen and antibody are present, the latter in such excess that it can be detected, and a positive reaction results.

Case 5.—Both are present, but there is also an inhibiting substance which interferes with the reaction of fixation, and therefore the result is negative.

Case 6.—The antigen formerly present has disappeared and no more is being liberated, but the antibody evoked by it still remains, being demonstrable in decreasing amounts on successive examinations.

Considered in the light of these hypothetical possibilities, the observed discrepancies in the result in examining a series of patients become more reasonable and susceptible of interpretation.

hope was promised of practicable employment of the method. The more recent work,[1] however, indicates an agreement of opinion upon the following points: The complement-fixation test gives a positive reaction in tuberculous infections only. The non-specific fixations with syphilitic serums reported by some investigators were probably due to imperfect fixing reagents or to a coincident infection with tuberculosis.

The von Pirquet and complement-fixation tests do not run parallel. Many of the incipient or normal cases having a positive tuberculin reaction give no fixation of complement. If a normal case having a positive von Pirquet gives a positive complement-fixation reaction, this should not be considered to be a "false positive," but the case should be kept under observation for the appearance of clinical symptoms.

With a suitable fixing reagent a positive complement-fixation reaction will be found in from 75 to 95 per cent. of clinically active pulmonary cases.

A negative complement-fixation reaction in any stage of the disease does not invalidate the test, as it may have great significance for prognosis and treatment.

Studies in this laboratory corroborate the above conclusions in regard to specificity, the discrepancy between von Pirquet and complement-fixation reactions and the incidence of positive reactions in clinically positive cases. Further than that, we have found that the fixing reagents made from the whole bacillus, either moist suspensions or defatted dried bacillus suspensions, are superior to any of the extracts we have tried. We had no fixation with alcoholic extracts.

The observation of von Wedel[2] that a clinically positive case giving a negative reaction twenty-four hours after bleeding may give a positive reaction on the seventh day after bleeding has been corroborated by us in a study of 500 cases; but we have not been able to prove that this has any diagnostic significance. Further study will have to determine the specific value of this "later" reaction.

Recent studies carried out at Trudeau Sanatorium have called attention to the value of the complement-fixation test in the management of the life of a tuberculous patient. A patient having a negative complement-fixation test can be allowed to exercise much more freely than a patient who has a positive complement fixation.

McIntosh and Fildes,[3] using an emulsion of fresh, living tubercle bacilli, found 80.7 per cent. complement-fixation reactions in orthopedic cases and 37.5 per cent. positive in glands.

In this laboratory, using the defatted fixing reagent described above, we have found 22 per cent. positive in bone and joint diseases and 58 per

[1] Kolmer: Infection Immunity and Biology Therapy, 3d ed., 1923, p. 554.
[2] Jour. Immunol., 1920, No. 5, **3**, 159.
[3] Lancet, 1914, **185**, 485.

cent. positive in glands. Keller and Moravek[1] state that they can differentiate between human and bovine infection in orthopedic cases, but we have not had that experience.

Bang and Anderson report a series of tests with tuberculous and non-tuberculous cattle. They state that strong reactions have diagnostic significance.

Very little work has been reported upon complement-fixation tests on cattle, and those reports indicate that the methods employed were not perfectly balanced.

Streptococcus Infections.—The value of complement fixation in the diagnosis of streptococcus infections is still uncertain. The test seems to be specific, but is far from perfected.

Pertussis.—Complement fixation has been used for the determination of the etiological cause of whooping-cough and for the diagnosis of the disease. The results of many investigators, including ourselves, confirm the findings of Bordet, that the Bordet-Gengou bacillus is the etiological cause of whooping-cough. As to the diagnostic value of the test, reports vary. The figures given by the workers who use active serum are undoubtedly too high, as investigations in this laboratory have proved that active serum may give non-specific fixation with even a highly specific antigen.

The Complement-fixation Test for Glanders.—Complement fixation has proved to be a valuable aid in the diagnosis of glanders. It is generally considered specific and more reliable than the agglutination test. The New York Health Department condemns all horses that give a + + + + complement-fixation reaction when it is confirmed by the eye mallein test, while those that give a strongly + + and + + + are suspected of having a slight glanders infection.[2]

Complement fixation has been successfully carried out in meningitis, typhoid, diphtheria and some of the parasitic skin[3] diseases, but is not of practical value in those diseases, as other and simpler laboratory tests are available for diagnosis.

[1] Int. Jour. Surg., July, 1915.
Other references are:
Besredka: Compt. rend. Acad. d. sc., 1913, **156**, 1633; Ztshr. Immunol., 1914, **8**, 931.
Bronfenbrenner: Arch. Int. Med., 1914, **14**, 786; Soc. Exp. Biol. and Med., 1914, 11.
Craig: Am. Jour. Med. Sci., 1915, **150**, 781.
Stimson: Bull. Hyg. Lab. U. S. P. H., 1915, No. 101, 7.
Petrcf: Am. Review of Tuberc., November, 1918, **2**, 9.
Miller: Proc. Soc. Exp. Biol. and Med., 1916, **13**, 134; Jour. Am. Med. Assn., November 18, 1916.
Wilson: Jour. Immunol., No. 5, vol. **3**.
Lange: Am. Jour. Tuberc., 1918, No. 9, vol. **2**.
Stoll and Neumann: Jour. Am. Med. Assn., 1919, **72**, No. 15.
Bordet and Gengou: Compt. rend. Acad. d. sc. Biol., 1903, **137**, 351.
Caulfield: Jour. Med. Res., 1911, **24**, 122.
Laird: Jour. Med. Res., 1912, **27**, 163.
Hammer: München. med. Wchnschr., 1912, **59**, 1750.
Calmette and Massol: Compt. rend. Soc. de biol., 1912, **75**, 120.
Burns, W. B.: Jour. Am. Med. Assn., **68**, 1386.
Corper: Jour. Infec. Dis., **19**, 315.

[2] At autopsy, macroscopic lesions are always shown by horses giving a + + + + complement-fixation test, rarely by those giving a + + or a + + + reaction.

[3] Kolmer and Strickler: Complement Fixation in Parasitic Skin Diseases, Jour. Am. Med. Assn., 1915, **64**, 800.

The Wassermann Reaction (The Complement-Fixation Test for Syphilis).—When Wassermann and Brück applied this test to the diagnosis of syphilis they used an alcoholic extract of syphilitic liver. They supposed that they were using an extract of the causative agent of the liver lesions. When, however, other workers obtained similar results with the alcoholic extracts of normal organs the original idea of immunological specificity had to be abandoned. When the Treponema pallidum was cultivated it was expected that a fixing reagent of this organism would give satisfactory results. This was not the case. More positive results were obtained with normal organ extracts than with a fixing reagent from cultures of the Treponema pallidum.

That is, the test is *disease-specific* as a diagnostic means; it is apparently *non-specific in an immunological sense,* as fixation is obtained with materials distinct from the causative agent or specific antigen of the disease.

Evidently, therefore, to term the fixing reagent an antigen is a wide departure from the etymological meaning of the term. This misuse leads to confusion and the term fixing reagent should be substituted.

Mechanism of the Wassermann Reaction.—The fixing reagents employed are essentially lipoidal extracts (oleates, lecithins and cholesterol).[1] Citron has applied the term "Lues reagin" to substances in which the blood volume, combined with the lipoidal reagent, fix complement. There is a physical change in syphilitic serums (and cerebrospinal fluid) that is noteworthy, namely the high content of globulin. The fixing substance or "Lues reagin" is in the globulin. The most one can say is that globulins are increased in syphilis, that they have a high affinity for the lipoids of the reagent and that the combination fixes complement.

Qualitative Test of Serum for Syphilis.—*Wassermann Diagnostic Test.*—The reagents employed in this test are similar to those in bacterial complement-fixation.

Reagents employed:

1. Patient's serum.
2. Fixation reagent (lipoidal antigen).
3. Complement.
4. Erythrocytes.
5. Hemolysin.
6. Saline.

The methods of obtaining and standardizing the reagents 1, 3, 4, 5 and 6 are given under microbal complement-fixation, pages 234 to 241.

Fixation Reagents (Antigens) for the Wassermann Reaction.—The methods given below are those which are generally employed for the test.

Crude Alcoholic Extract Lipoidal Fixing Reagent.—Guinea-pig hearts are minced and washed in tap water until free from blood. The minced hearts are macerated in ether; then are washed three times in 95 per cent. alcohol, and again in ether. The minced material is then placed in a sterile dish and allowed to dry at room temperature in the dark. This usually takes from three days to

[1] See Kahn test for a similar disease-specific flocculation reaction, which reaction, however, is not immunologically specific as are the precipitin reactions described in the chapter on Precipitins.

one week, depending upon the number of hearts used. The dried heart material is ground in a mortar with absolute alcohol, in the proportion of 1 gram of heart to 5 c.c. of alcohol. This alcohol-heart mixture is poured into a sterile bottle and placed in the incubator at 37° C. for one month for extraction. The bottle is shaken thoroughly once or twice every day. The mixture is then filtered through filter paper and the filtrate is stored in sterile bottles.

Cholesterinized fixing reagent may be prepared from the crude alcoholic heart fixing reagent described above. This crude alcoholic fixing reagent is divided into two parts. The first part is set aside. The second part is heated in a water-bath to 40° C. and cholesterol crystals are added to saturation (about 0.8 per cent.). It is now placed in the incubator at 37° C. for twenty-four hours, to insure saturation, and then cooled to room temperature. This will precipitate out excess cholesterol, which is removed by filtration. The saturated cholesterinized fixing reagent is now combined in equal volumes with the first part of the crude alcoholic fixing reagent. Half saturation with cholesterol results, and the mixture constitutes the finished cholesterinized fixing reagent. It should be standardized and appropriately diluted for use. Such fixing reagents frequently show a tendency to be oversensitive in binding complement, especially in ice-box fixation. For this reason, in performing actual tests where they are used, the method of fixation should be that of one hour at 37.5° C. (water-bath).

Noguchi's acetone insoluble fraction of beef heart, liver, or kidney, may be prepared by extracting macerated tissue with ten times the amount of absolute alcohol at room temperature for several days, filtering, evaporating the filtrate to dryness, taking up the residue with ether, treating the ethereal solution with five times its volume of acetone, and making a saturated solution of the precipitate in absolute methyl alcohol.

Bordet and Ruelen's Acetone-insoluble Alcoholic Fixing Reagent.[1]—Remove the fat, bloodvessels, pericardium and endocardium, of beef heart and cut up the muscle into cubes about 1 inch in diameter. Then grind the heart muscles in a meat chopper. Weigh the ground tissue, distribute 100 gm. amounts in clear glass flasks and to each add 125 c.c. of 95 per cent. alcohol. (The purpose of the alcohol is to coagulate the proteins and not to dissolve the fatty substances or lipoids, and as the alcohol is in comparatively small proportion and is immediately diluted with the tissue juice, it exerts but slight dissolving power. Let the mixture stand at room temperature for several days, then filter, spread the residue on trays in a thin layer and dry in the incubator for a day. Distribute the tissue equally among the same number of flasks originally used and to each add 200 c.c. of acetone. Mix and leave at room temperature for one week, shaking for a minute or two once a day, then filter. Add 200 c.c. of fresh acetone to the tissue in each flask, mix and filter the next day. Remove the tissue to trays and dry in the incubator for one day, then pulverize it finely in a mortar. Distribute the ground tissue equally among the number of flasks originally used and to each add 200 c.c. of 95 per cent. alcohol. Mix and allow to extract at room temperature for ten days, shaking for a minute or two three times a day. Then remove the alcoholic extract by filtration through soft filter paper, and *store in the dark at room temperature.*

This last fixing reagent has given such satisfactory results that it is now used at the New York State and New York City laboratories instead of the "crude alcoholic" reagent.

Standardization of the Fixing Reagents.—The fixing reagents for the Wassermann reaction are standardized in the same manner as the bacterial fixing reagents. They must answer the same requirements as to range and specificity. Special emphasis is placed on the specificity of this reagent; since this fixing reagent is in its nature non-specific with regard to the syphilitic antibody in the patient's serum. Therefore special care must be taken that the reaction should be *disease-specific.* In other words it must be so standardized that it will not

[1] Bordet, J., and Ruelens, G.: l'Antigene syphilitique de l'Institut Pasteur de Bruxelles, Compt. rend. Soc. de biol., 1919, **82**, 880.

fix complement when combined with complement-fixing antibodies of other diseases.

In the standardization tests, the serum of known syphilitic patients is used as the immune serum. The number of fixation units in the serum is determined by an antibody titration (page 247) and then two to four fixation units are used to standardize the fixing reagent. For the method of titration see page 247.

Standardization of Complement.—Earlier in this chapter are described three forms of complement-fixation tests, namely the qualitative diagnostic test, the quantitative diagnostic test and the antibody content titration of immune animal serums. The standardization of the complement varies in some respects in those different tests and for the sake of simplicity we have given the exact procedure for each case under its appropriate heading.

Standardization of Complement for the Qualitative Diagnostic Test for Syphilis (Wassermann Test).—After the guinea-pigs have been killed, the serum removed from clots and freed from cells, as described on pages 238 and 239 the serum from each pig is kept separate until the complement has been standardized. Certain preliminary tests of the individual pig-serums are essential before reliable diagnostic tests can be performed. We must know whether the complement is free from natural antisheep amboceptor, and whether the complement has average hemolytic activity. These questions are answered in our preliminary tests Nos. 1 and 2.

The complement is then pooled and titrated for the hemolytic unit (see page 240). For routine diagnostic tests it was found unnecessary to test each individual pig for fixation as the fixing reagent used for the tests gives uniformly good results with pooled guinea-pig serum. But after pooling, the complement is tested for fixation in the same manner as for the individual pig.

The Kind of Fixing-reagent, Temperature and Period of Incubation for the Wassermann Reaction.—1. Bordet and Ruelen's acetone-insoluble reagent fixation: Four hours at 8° to 10° C.

2. Cholesterinized alcoholic fixation reagent. Fixation: One hour at 37° C.

The Qualitative Complement-fixation Diagnostic Test.—*Reagents:* Salt solution (see page 235), erythrocytes (see page 235); hemolysin (see page 236); complement (see page 238); antigen (see pages 242–244); patient's serum (see page 235).

Purpose of the Qualitative Test.—To determine whether antibody for the etiological organism of the disease in question is present in the patient's serum. (When the test is made with the bacterial fixing reagent.) Or, to demonstrate the presence in the patient's serum of syphilitic reagin. (When the test is made with lipoidal fixing reagent.)

Method.—To determine whether a known dose of complement will be fixed. The fixation of such a dose of complement indicates the presence of antibody in the patient's serum.

Before beginning the diagnostic tests the reagents must all be prepared and standardized in accordance with the methods described on the pages indicated above. The constant reagents: hemolysin, fixing reagent and erythrocytes, are always used in the standard dilution.

For the methods described here it is most convenient to pipette the reagents as follows: Measure fixing reagent and complement dilutions with 1 c.c. pipettes graduated into 0.1 c.c. Measure serums with 0.2 c.c. pipettes graduated into 0.01 c.c. Measure sensitized cells with 2 c.c. pipettes, graduated into 0.1 c.c.

The reactions are read more easily if the tests are made in test-tubes measuring 3 inches in height by $\frac{3}{8}$ inch in diameter.

Operations of the Test.—1. Standardization of the complement; 2, the qualitative test.

1. The variable reagent, complement, must be titrated every day to determine its reaction with the known reagents. The exact method for standardizing complement for the qualitative diagnostic test for syphilis (with lipoidal fixing reagent) is given on page 256. The method for standardizing complement for the qualitative test with bacteria fixing reagent is given on page 241. With the exception of the difference in standardizing the complement, and the time and temperature for fixation of complement, the qualitative diagnostic test with lipoidal fixing reagent and with bacterial fixing reagent is the same. The time and temperature for fixation are given on page 247.

2. Having found the correct dose of complement, the diagnostic test and all controls[1] are pipetted in accordance with the following table:

TEST OF SERUM FOR DIAGNOSIS, SETTING UP THE DIAGNOSTIC TEST IN DUPLICATE.

Tube No.		Patient's serum undiluted, c.c.	Fixing reagent standard dilution, c.c.	Complement standard dilution, c.c.	Saline solution .9 per cent.		Sensitized cells.	Five per cent. suspension of erythrocytes.
Serum a/c control	1	.04	.0	.1	.06	Fixation. (See pages 247 and 248.)	.2	.0
	2	.02	.0	.1	.08		.2	0
Diagnosis test in duplicate	3	.02	.1	.1	.08		.2	0
	4	.01	.1	.1	.09		.2	0
	5	.02	.1	.1	.08		.2	0
	6	.01	.1	.1	.09		.2	0
Fixing reagent a/c control	7	0	.4	.1	0		.2	0
	8	0	.3	.1	0		.2	0
	9	0	.2	.1	0		.2	0
	10	0	.1	.1	.1		.2	0
System control	11	0	0	.1	.2		.2	0
Cell control	12	0	0	0	.3		.2	0
Natural-antisheep amboceptor control	13	.04	0	.1	.16		.0	.1
	14	.02	0	.1	.18		0	.1
	15	.02	.1	.1	.18		0	.1
	16	.01	.1	.1	.19		0	.1

In these tables "a/c" means anticomplementary.

The tests and all controls are placed for fixation of complement in accordance with the method for the disease in question, as described on pages 247 and 248.

Reading and Interpreting the Test.—After the period of fixation is completed a standard amount (indicated in the different tables) of sensitized erythrocytes is added to all tubes. The tests are then placed in a water-bath at 37° C. until the fixing reagent control and serum control tubes show complete hemolysis.[2] Readings of the degree of complement fixation in each tube of the test are then made.[3]

[1] In addition to the controls described in the above table, there should be included in each day's tests a known positive serum and a known negative serum. The latter is a control of the specificity of the fixing reagent with this lot of complement, and the former is a control of the stability of the complement. For the former, we prefer to make a complete fixing-reagent titration.

[2] With our system the hemolytic controls are hemolyzed in ten minutes.

[3] See table on page 258.

Complement-fixation Reaction, Diagnostic Interpretation.—Having performed the test and controls, in accordance with the foregoing schemes, we have enough data to assure us that the reactions shown in Tubes 3 and 4 are true fixing-reagent-antibody reactions or fixing-reagin reactions. The next step is to make the complement-fixation diagnosis. Citron's standard for the strength of a reaction is followed, with modifications when necessary.[1] The following is Citron's standard for laboratory diagnosis.

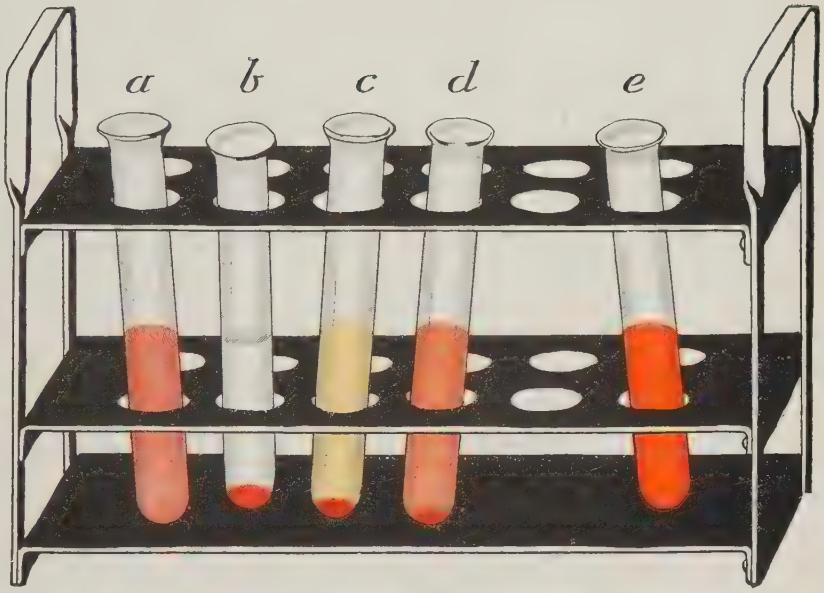
INTERPRETATION OF COMPLEMENT-FIXATION REACTIONS IN TEST AND CONTROLS.

Tube No.	Purpose of test.	A Reactions indicating a true positive.	B Reactions indicating a true negative.	C Reactions indicating a false positive.	D Reactions indicating a false negative.
1	Control of a/c reaction	Completely hemolyzed	Completely hemolyzed	Completely fixed	Completely hemolyzed.
2	Of patient's serum	Completely hemolyzed	Completely hemolyzed	Completely fixed	Completely hemolyzed.
3	Original test	Completely fixed	Completely hemolyzed	Completely fixed	Completely hemolyzed.
4	Original test	Completely fixed	Completely hemolyzed	Completely fixed	Completely hemolyzed.
5	Duplicate test	Completely fixed	Completely hemolyzed	Completely fixed	Completely hemolyzed
6	Duplicate test	Completely fixed	Completely hemolyzed	Completely fixed	Completely hemolyzed.
7		Trace of fixation	Trace of fixation	Trace of fixation	Trace of fixation.
8		Completely hemolyzed	Completely hemolyzed	Completely hemolyzed	Completely hemolyzed.
9	Control of a/c reaction of fixing reagent	Completely hemolyzed	Completely hemolyzed	Completely hemolyzed	Completely hemolyzed.
10		Completely hemolyzed	Completely hemolyzed	Completely hemolyzed	Completely hemolyzed.
11	Control of complement activity	Hemolyzed in 10 minutes	Hemolyzed in 10 minutes	Hemolyzed in 10 minutes	Hemolyzed in 10 minutes.
12	Stability of cells; reading control	No hemolysis	No hemolysis	No hemolysis	No hemolysis.
13	Control for natural antisheep amboceptor in patient's serum	No hemolysis	No hemolysis	No hemolysis	Completely hemolyzed.
14		No hemolysis	No hemolysis	No hemolysis	Completely hemolyzed.
15		No hemolysis	No hemolysis	No hemolysis	Completely fixed.
16		No hemolysis	No hemolysis	No hemolysis	Completely fixed.
Complement-fixation diagnosis		4+	Negative	No diagnosis. Patient's serum is anticomplementary	4+ Naturalamboceptor in patient's serum interfered with fixation test.

Column (A) represents the reactions which are interpreted as a 4+.
Column (B) represents the reactions which are interpreted as a negative.
Column (C) represents false positive reaction in test due to an anticomplementary patient's serum.
Colum (D) represents a false negative (occurring rarely) due to an excess of amboceptor. Tubes (13) and (14) contain same reagents as tubes (1) and (2); tubes (15) and (16) same as (3) and (4), except that after the fixation period no artificial amboceptor is added to (13), (14), (15) and (16). Therefore the hemolysis in (13) and (14) is due to natural antisheep amboceptor in patient's serum. A true 4+ diagnosis can be made upon the control (13), (14), (15), (16).

[1] The following reaction is not mentioned by Citron, but is encountered so frequently in our tests that we have included it in our diagnostic reactions: Tubes 1 and 2 both show very strong fixation, but not complete fixation. The 0.01 amount in Tube 2 is as strong as in a three-plus reaction, and the 0.02 amount in Tube 1 is only slightly weaker than in a three plus. We have termed this reaction "strong one plus." It is stronger than a two plus and very slightly weaker than a three plus. Therefore, we report it as a strongly positive reaction.

PLATE V



a. Complete absence of hemolysis before settling of cells.
b. Complete absence of hemolysis after settling of cells.
c. Partial hemolysis.
d. Nearly complete hemolysis.
e. Complete hemolysis.

(*a*) Tubes 1 and 2 show complete absence of hemolysis. ++++	Strongly Positive.
(*b*) Tube 1 shows complete absence of hemolysis and 2 shows faint hemolysis. +++	
(*c*) Tube 1 shows complete absence of hemolysis and 2 shows partial or complete hemolysis. ++	Positive.
(*d*) Tube 1 shows partial hemolysis, and 2 complete hemolysis. +	Weakly Positive.
(*e*) Tube 1 shows doubtful binding and 2 complete hemolysis. ±	Doubtful.
(*f*) Tubes 1 and 2 show complete hemolysis. −	Negative.

Qualitative Test of Spinal Fluids for Syphilis.—When testing spinal fluids for syphilis, the fluid is not inactivated, and it is pipetted in the original Wassermann volumes of 0.2 and 0.1 c.c., while all the reagents are used in the 0.5 volumes as in the test of serum.

TEST OF SPINAL FLUID FOR DIAGNOSIS OF SYPHILIS.

Number of tube.	Patient's spinal fluid, c.c.	Fixing reagent in standard dilution, c.c.	Ten per cent. complement, c.c.	0.85 per cent. saline, c.c.	Period for fixation (4 hours in ice-box at 8° C.)	Sensitized erythrocyte suspension, c.c.	Incubated (water bath 37.5° C.) until hemolysis is complete in tubes 5 and 6.
1	.2	.1	.1	.1		.25	
2	.1	.1	.1	.1		.25	
3	.2	.1	.1	.1		.25	
4	.1	.1	.1	.1		.25	
5	.2	0	.1	0		.25	
6	.4	0	.1	0		.25	
7	.08	.1	.1	.2		.25	
8	.06	.1	.1	.2		.25	
9	.04	.1	.1	.2		.25	
10	.02	.1	.1	.2		.25	
11	.01	.1	.1	.2		.25	

N. B.—Spinal fluid is always used without being inactivated.

Reading and Interpreting Tests on Spinal Fluid.—In testing spinal fluid for syphilis it is customary to use relatively large amounts (ten times as much fluid as serum). (See Table above.)

Because of the large number of the different amounts of spinal fluid used in the test, a reading and interpretation according to Citron's standard is not possible. As most spinal fluids are from patients who have already been diagnosed as syphilitic, complete fixation of complement by the fixing reagent and any amount of spinal fluid (as used above) is considered a positive test; partial fixation by the fixing reagent and any of the larger amounts of spinal fluid is considered a doubtful reaction; while complete hemolysis in all amounts of fluid is, of course, a negative reaction. If a series of tests similar to the above be carried out on spinal fluid withdrawn at different intervals from a patient over a prolonged period of time, and if the exact amount of inhibition of hemolysis in each tube be recorded in each test and compared with the degree of inhibition in the corresponding tubes in the succeeding tests

of the series, such a scheme may be of value in observing and following the course of treatment.

The Present Status of the Complement-fixation Test in the Diagnosis of Syphilis.—According to most workers, there has been enough study of this test (Wassermann test) to establish its value as a diagnostic measure. Some interesting points have been brought out in connection with this study, and many different methods have been recommended. Though biologically non-specific, the Wassermann reaction is clinically specific, except perhaps, when certain methods are employed in cases of leprosy, yaws and sleeping sickness. It has been claimed that non-specific reactions have been obtained with serums from cases of scarlet fever, tuberculosis and some other diseases, but these claims have not been proved. Hard and fast statements in regard to non-specific reactions cannot be accepted until the clinician is able to give us more reliable histories of the patients. The test has been established as a routine diagnostic measure in all public health laboratories and in most hospitals.

In an immense number of cases (90 to 95 per cent. of cases with active general infection) positive results have been obtained. A positive reaction (with the exceptions noted above) indicates the presence of luetic infection, either active or latent. A negative reaction at any stage of the disease does not exclude the possibility of syphilis. In general paralysis and in the majority of cases of tabes a positive reaction occurs in the tests of blood serum and also of the spinal fluid. The reaction is practically always positive in the secondary stage of syphilis. The reaction gradually disappears when the disease becomes inactive or is cured. It may disappear before a cure is established, to reappear if an active process starts.

Treatment with arsphenamin and even with mercury and the iodides may at first or after a few treatments cause a negative Wassermann to become positive for a time. A cure can be pronounced when the reaction has remained negative for at least a year after intensive treatment and when it then still remains negative after a provocative treatment with arsphenamin.

The ingestion of alcohol or the administration of an anesthetic within twenty-four hours before the collection of the blood specimen may interfere with the accuracy of the test, alcohol weakening the reaction and an anesthetic giving rise to false positive reactions.

Spinal fluids may give a positive reaction when the blood serum is negative in cases in which the brain or cord is involved. Hence the spinal fluid should always be tested in addition to the serum, if that is negative, whenever syphilis of the nervous system is suspected.

The addition of cholesterol to the extent of half-saturation to a crude alcoholic extract fixing reagent has been recommended by some serologists. Such a fixing reagent gives a higher percentage of positive reactions than the crude extracts, but its reliability is still in question by some workers, at least for primary diagnosis. We have found the cholesterinized fixing reagent to be of special value in determining the

effectiveness of antiluetic treatment, as old cases of syphilis that are or have recently been under treatment are more liable to react with this fixing reagent than with crude alcoholic fixing reagent. But in primary diagnoses we find it unreliable and, therefore, do not use it at present.

The Application of the Complement-fixation Reaction to the Identification of Proteins.—The technic of the test is essentially the same as for bacterial complement-fixation. The immune serum is obtained by immunizing animals with the protein in question. The protein is then used as the fixing reagent (antigen). The reaction is specific and is used to identify unknown proteins.

The Kahn Precipitation Test for Syphilis.—*History.*—Some interest in precipitation tests for syphilis in which tissue extracts are used as precipitin reagents began soon after Wassermann, Neisser and Brück published the Wassermann test. Michaelis,[1] in 1907, published a precipitation test for syphilis in which he used a watery extract of syphilitic liver as an antigen. Three years later Jacobstahl[2] published a precipitation method in which the ultramicroscope was used in recognizing the precipitates. This was followed by tests proposed by Brück and Hidaka[3] in 1911 and by Hecht[4] in 1914. In 1914 Olitsky and Olmstead[5] of our laboratories were unsuccessful in their attempt to obtain reliable precipitation in syphilis with alcoholic extracts of guinea-pig heart. In 1917 Meinicke[6] published the so-called water and salt solution precipitation methods which appear to have awakened a new interest in the serum diagnosis of syphilis by precipitation. In 1919 Meinicke[7] published the Third Modification which he claimed to be a combination of his two earlier procedures. The precipitation reagent in this method consists of an alcoholic extract of dried horse heart previously extracted with ether. The employment of 2 per cent. sodium chloride solution appears, according to this author, to play an important rôle in this reaction. One year previous to Meinicke's third publication, Sachs and Georgi[8] published the test which now bears their names and is probably the most widely known of all precipitation tests, particularly in Germany. In 1921 Dreyer and Ward[9] published a quantitative precipitation test known as the Sigma reaction, which is receiving some recognition in Great Britain. Since then Hecht[10] and Bruck[11] modified their original methods, and there has also been published a precipitation test by Wang[12] and a turbidity test by Dold.[13] Meinicke[14] published also, two turbidity tests: one with a cholesterinized precipitation reagent and another with a cholesterin-free precipitation reagent. More recently, the Vernes test has received considerable attention.[15,16] The work of those and other authors constitute a large bibliography.

Nature of the Precipitation Reaction.—The precipitation test for syphilis is not a reaction by precipitinogen and precipitin, but is a non-specific colloidal reaction. The mechanism of the precipitation reaction is not fully understood, although several attempts have been made to explain it. Epstein,[17] Scheer and

[1] Berl. klin. Wchnschr., 1907, **44**, 1477.
[2] Ztschr. f. Immunitatsforsch. Orig., 1910, **8**, 107
[3] Ibid., 1911, **8**, 476.
[4] Ibid., 1915, **24**, 258.
[5] Jour. Am. Med. Assn., 1914, **62**, 293.
[6] Berl. klin. Wchnschr., 1917, **54**, 613.
[7] München. med. Wchnschr., 1919, **66**, 632.
[8] Med. klin., 1918, **14**, 805.
[9] Lancet, 1921, **1**, 956.
[10] Arch. f. Dermat. u. Syph. Orig., 1921, **136**, 296.
[11] München. med. Wchnschr., 1922, **69**, 569.
[12] Lancet, 1922, **1**, 274.
[13] Med. klin., 1921, **17**, 940.
[14] Deutsch. med. Wchnschr., 1922, **48**, 384 and Ibid., 1923, **49**, 43.
[15] Arch. Derm. and Syphil., 1922, **5**, 433.
[16] Proc. Soc. Exp. Biol. and Med., 1923, **21**, 1.
[17] Deutsch. med. Wchnschr., 1922, **48**, 89–91.

Neiderhoff made a chemical analysis of the precipitate and found it to be almost entirely of a lipoid nature.

Kahn's Method for Precipitation in Syphilis.—Early in 1922 Kahn published a modification of Meinicke's test. The outstanding feature of Kahn's method is the element of concentration of the precipitation reagent in contradistinction to practically all other precipitation methods in which the high dilution of the reagent with salt solution plays an important rôle. In seeking to produce a reagent of high concentration, Kahn observed that an alcoholic extract of powdered beef heart, previously freed from ether soluble elements, gives a reagent superior to other alcoholic extracts. Kahn's method for preparation of precipitation reagent is similar to Meinicke's and practically the same as Neumann and Gager's Wassermann reagent.

Recently Kahn has modified his test in several details.[1] By increasing the concentration of his precipitation reagent in the saline mixture he was able to practically eliminate from the test the necessity for incubation.

The precipitation-reagent salt solution mixture used in the test contains a heavy precipitate. Bur this precipitate immediately goes back into solution when it comes in contact with serum. A negative serum remains clear, but a syphilitic serum soon begins to show the presence of a new precipitate. Another observation in connection with this procedure is that varying amounts of syphilitic substance require varying amounts of the precipitation reagent to bring about optimum precipitation reactions. This necessitates the use of three tubes with different precipitation reagent proportions for each test. The final result is the average find of the three tubes. This test has been studied in the New York City Health Department Laboratories,[2] and the results are sufficiently promising to justify, in our opinion, detailed presentation of the method in this text-book.

Technic of Kahn's Method.—The reagents used in this test are: (1) Salt solution; (2) patient's serum; (3) precipitation reagent.

The reaction of the glassware as well as the preparation of the physiological salt solution is the same as described under Complement-fixation Test for Syphilis (page 235). Sterility of salt solution is not necessary, but chemical purity is essential. Only distilled water should be used in the preparation of salt solution and it should be filtered to free it from foreign particles.

The patient's serum is prepared as described on page 235. It should be inactivated for thirty minutes at 56° C. before using in the test. Serums that have remained in the ice-box overnight after being inactivated should be reheated for five minutes at 56° C.

Chylous serums and hemolyzed specimens, if not excessive, may be used. It is not uncommon for apparently clear, raw serums to show the presence of a weak precipitate after heating. In such a case, the precipitate should be removed by centrifugalization.

Preparation of the Precipitation Reagent.—1. *Powdered Muscle.*—Beef hearts as fresh as possible are freed from fat, fiber and bloodvessels. The heart muscle

[1] The Kahn Test, a Practical Guide, Baltimore, Williams & Wilkins Company, 1928.
[2] Jour. Lab. and Clin. Med., 1924, No. 9, vol. 9.

is cut into fine pieces and passed through a meat grinder three times. The ground material is then spread in a thin layer on a platter and dried by means of one or two revolving fans. After six to eight hours' drying, the material is turned over and the drying continued for ten hours or over night. The comparatively dry layer is broken into small pieces with the hands and the drying is continued until the particles are crisp. The dried pieces are powdered in a mortar, or by grinding several times in a coffee mill that is not used for any other purpose.

An average sized beef heart will produce several hundred grams of powdered muscle, and 50 grams of the latter will yield about 150 c.c. of precipitation reagent. A number of beef hearts should be used in preparing the precipitation reagent rather than one; as different beef-hearts yield precipitation reagents of varying sensitivity.

2. *Extraction with Ether.*—Twenty-five grams of powdered beef heart are placed in a 250 c.c. Erlenmeyer flask. One hundred c.c. of ether (anesthesia) are added and the flask is shaken at frequent intervals for ten minutes. At the end of this period the ether is filtered off. Gentle pressure is applied to the beef heart in the funnel by means of a spatula, to assure as complete removal of the ether as possible. The filtration is completed when further pressure does not cause drops of ether to pass through the funnel. The moist beef heart is now transferred to the original flask and 75 c.c. fresh ether added. The flask is again shaken at frequent intervals during a ten-minute period, and the ether filtered off as in the previous case. The heart muscle is returned to the flask a third time and again covered with 75 c.c. ether. The flask is shaken from time to time during a ten-minute period and filtration carried out as previously.

The moist powder is now transferred to the Erlenmeyer flask for the fourth and last ether extraction. Seventy-five c.c. ether are added to the flask, the mixture shaken for ten minutes, and final filtration carried out. The first three ether filtrations may be carried out with the same filter paper. For the last filtration, however, a fresh layer of filter paper is employed. After the ether is removed as completely as in the earlier filtrations, the moist heart muscle is spread upon a white sheet of paper or a clean glass plate and dried in the incubator at 37° C. for about ten minutes or for a somewhat longer period at room temperature. When the material is dry and free from ether odor, it is ready for extraction with alcohol.

3. *Extraction with Alcohol.*—The ether extraction being completed, the powdered muscle is reweighed and placed in a 250 c.c. flask. An amount of 95 per cent. alcohol is then added in the proportion of 5 c.c. of alcohol per gram of powder. The mixture is thoroughly shaken for ten minutes and extracted for three days at room temperature (21° C.) without shaking. At the end of this period it is shaken for five minutes and the alcoholic extract is filtered off. This extract is kept at room temperature in the dark in tightly corked flasks. Rubber stoppers should not be used in connection with the preparation and storing of the reagent, and corks should be covered with tin foil.

4. *Cholesterinization of Alcoholic Extract.*—An amount of alcoholic extract which is likely to be used within one to two months is measured into an Erlenmeyer flask and cholesterin added in the proportion of 6 mg. per c.c. The cholesterin is dissolved by rotating the flask in a water-bath or by placing the flask for some hours in the incubator. When all of the cholesterin has been dissolved, the solution is filtered to free it from foreign particles. This extract is also kept at room temperature in the dark.

Standardization of the Precipitation Reagent.—The cholesterinized extract is titrated with salt solution to determine in what proportions to dilute the reagent with salt solution for the diagnostic tests. More specifically, the titration aims to find the smallest amount of salt solution to add to the reagent which will produce a precipitate capable of dissolving on further addition of salt solution. Since the manner in which salt solution is added to reagent has a marked effect on the solubility of the resulting precipitate, it is important in this regard to follow the directions to be outlined. For simplicity, a titration method with

1 c.c. amounts of reagent will be given, although other quantities may be used, provided the proportional salt solution amounts are carefully noted.

Method.—To each of five tubes of 1 cm. diameter and 7.5 cm. length is added 1 c.c. of cholestrolized reagent. To five similar tubes are added 0.6, 0.8, 1, 1.1 and 1.2 c.c. salt solution respectively. Each quantity of salt solution is mixed with one of the reagent amounts by pouring the salt solution into the reagent tube and immediately pouring the mixture back and forth several times. Each of the five tubes will show a heavy precipitate, but the solubility of these precipitates will vary markedly. Thus, the precipitate resulting from 1 c.c. reagent and 0.6 c.c. salt solution will be found to be insoluble in salt solution, whereas the precipitate resulting from 1 c.c. reagent and 1.2 c.c. saline will almost invariably be found to be readily soluble in salt solution. The element of time, however, has a marked influence on the solubility of these precipitates. The longer the interval after mixing the reagent with salt solution, the more insoluble the precipitate becomes. Because of this tendency, the reagent dilutions are permitted to stand for a half hour before testing the solubility of the precipitates.

The solubility tests are carried out as follows: Set up five series of three standard tubes. Pipette 0.05, 0.025, and 0.0125 c.c. quantities of each of the five reagent suspensions, in series, to the bottom of the tubes, using a 0.2 c.c. pipette graduated into 0.001 c.c. Add 0.15 c.c. quantities of normal saline to each of the 15 tubes and shake the tubes for three minutes in a shaking apparatus at a speed of 275 to 285 oscillations per minute. If no such apparatus is available, rapid shaking by hand will approximate this speed. Then add 1 c.c. salt solution to the tubes containing 0.05 c.c. amounts of the reagent suspension, and 0.5 c.c. to the remaining tubes. Observe whether the fluids are opalescent or contain aggregates.

The titer of the reagent is represented by the reagent dilution containing the minimum amount of salt solution which produces a precipitate capable of redissolving in salt solution.

TYPICAL PRECIPITATING REAGENT TITRATION FOR TEST WITH SERUM.

Reagent dilution series	1	2	3	4	5
Reagent + salt solution c.c.	1 + 0.6	1 + 0.8	1 + 1.0	1 + 1.1	1 + 1.2
Result of dilution	Heavy precipitate in each reagent dilution				
Scheme used in testing Solubility of precipitate in each reagent dilution	Tube No.		1	2	3
	*Reagent dilution c.c.		0.05	0.025	0.0125
	Salt solution c.c.		0.15	0.15	0.15
	Tubes are shaken two minutes and 0.5 c.c. salt solution added to each. All are observed for precipitates.				
Solubility of precipitate as determined by three-tube test	Precipitate not soluble	Precipitate not soluble	Precipitate soluble	Precipitate soluble	Precipitate soluble
Standard reagent dilution			Reagent + minimum amount of salt solution giving precipitate which dissolves in salt solution		

* Each reagent dilution is allowed to stand thirty minutes after mixing reagent and salt solution before solubility test is made.

Interpretation of Titration Results.—In this titration an amount of saline equal to that of precipitation reagent represents the smallest amount of saline which produces a precipitate capable of dissolving in saline one-half hour after mixing. This reagent, therefore, is diluted for the tests in the proportion of 1 + 1 with saline and the dilution is used with serum within one-half hour.

It should be added that precipitation reagent suspensions that are dispersed in additional saline are likewise dispersed in serum.

Determination of Sensitiveness of the Precipitation Reagent.—As precipitation reagents prepared from different lots of heart muscle are likely to vary in sensitiveness, it is necessary to adjust each new reagent to a certain standard. Standard reagents are now available in the market. The newly-prepared reagent and the standard are tested simultaneously with a number of syphilitic serums.

A reagent may be less sensitive than the standard either because the concentration of lipoids is too great or insufficient, and the method of adjusting it to standard would depend upon the above conditions. If the lipoid concentration is too great, the reagent is diluted with cholestrinized alcohol (0.6 per cent. cholesterol in 95 per cent. alcohol). If on the other hand the lipoid concentration is insufficient, the reagent is diluted with a sensitizing reagent until it comes up to standard.

A reagent that is too sensitive may be adjusted in three different ways: (1) by diluting with cholestrolized alcohol; (2) concentrating of lipoids; (3) adding saline beyond the requirement of titer.

Preparation of Sensitizing Reagent.—The ether filtrate obtained in the preparation of precipitation reagent from 50 grams of beef-heart muscle is refiltered and evaporated to dryness. The dry residue is extracted with absolute alcohol in the proportion of 10 c.c. alcohol per gram of residue. Extraction is continued for ten minutes at room temperature with frequent shaking. Then the mixture is placed in the ice-box (4° to 9° C.) for three hours. The mixture is filtered while cold, then incubated at 37° C. for twenty-four hours. The filtrate is left at room temperature for three days. If any precipitate forms during this time, the solution is filtered again and 0.6 per cent. cholesterin is added in the manner described in the preparation of precipitation reagents and the solution filtered and stored.

The Kahn Test.—*The Qualitative or Routine Diagnostic Test with Serum.*—Reagents required for the test:

1. Precipitation reagent previously standardized.
2. Patients serum inactivated for thirty minutes at 56° C.
3. Physiological saline.

The glassware necessary to carry out the test consists of test-tubes about 1 cm. in diameter by 7.5 cm. in length, 1 cc. pipettes, graduated in 0.01 c.c., and 0.2 c.c. pipettes, graduated in 0.001 c.c. Each test employs three tubes with varying amounts of serum and reagent dilution in accordance with the following scheme:

Tube No.	1	2	3
Serum: Precipitation reagent dilution	3:1	6:1	12:1
Precipitation reagent dilution c.c. .	0.05	0.025	0.0125
Serum (undiluted) c.c.	0.15	0.15	0.15

Preparation of Standard Precipitation Reagent Suspension.—Mix the reagent with physiological salt solution according to required titre. Thus, if the titre is 1 c.c. the reagent + 1 c.c. salt solution, mix the reagent as follows:

Measure 1 c.c. salt solution into a standard vial, and 1 c.c. of the reagent into a similar vial. Pour the salt solution into the reagent vial as rapidly as possible, and then back and forth six times to insure thorough mixing.

Allow the reagent suspension to stand ten minutes before using. The suspension should not be used after thirty minutes standing as the mixture is not stable and some of the precipitate may become insoluble after that length of time.

The Test.—When running a given number of tests, it is well to have the tubes set up and numbered and the serums inactivated and ready for pipetting, before diluting the reagent with the saline.

After the reagent suspension has stood for ten minutes, shake it well and measure 0.05, 0.025, and 0.0125 c.c. amounts into the tubes delivering each suspension to the bottom of the tubes. Add 0.15 c.c. serum to each of the 0.05, 0.025 and 0.0125 amounts of reagent suspension and shake vigorously for three minutes preferably in a shaking machine at uniform speed. Then add 1. c.c. saline to the tube containing 0.05 c.c. reagent suspension and 0.5 c.c. saline to the remaining tubes. Shake sufficiently to mix the ingredients, and read the results.

Controls.—A preliminary reagent control is carried out to insure against possible error in the amounts of reagent and salt solution employed, as well as against improper mixing. This is done by establishing that the reagent-saline precipitate is readily soluble in salt solution. About 0.05 c.c. of the reagent dilution is measured into a test tube and about 1 c.c. salt solution added and vigorously shaken for about ten seconds. There should be no trace of a precipitate.

Each serum is examined for particles which may give the appearance of a specific precipitate. Dilute 0.1 c.c. serum with 0.3 c.c. saline. This corresponds to the final dilution of the serum in the test. Shake the tube for three minutes and examine for particles. If necessary the serum is centrifugalized and tested again in the same manner.

Known positive and negative serums are included in the tests in order to insure specificity of the reaction; also a precipitation reagent control. This last consists of the three amounts of precipitation reagent used in the test to which are added 0.15 c.c. saline instead of serum.

If it is desired to hold the test for a given length of time for check purposes (after the addition of salt solution), it is well to keep the tubes in the ice-box.

Method of Reading and Interpretation of the Test.—The readings are made in front of a window with a darkened background. No difficulty is encountered in reading the strongly positive reactions. The tubes showing weaker reactions are read individually. Each tube is lifted above the eye level and slanted until it is practically horizontal. This causes the fluid to spread into a thin layer and renders the floating particles readily visible.

The tests are read on the basis of 1, 2, 3 and 4 plus. A tube showing a definite precipitate suspended in a clear medium is read 4 plus. Precipitates of less intensity are read 3, 2 and 1 plus, proportionally. Occasionally one will observe quantitatively fewer particles in the tubes containing the smaller amounts of precipitation reagent dilution compared with the larger amounts. But, if the nature of the particles is relatively the same, the tubes are read 4 plus in spite of the quantitative difference between the number of particles.

The final result in each test is the average reading of the three tubes. A reaction is 4 plus when the reading in each tube is 4 plus. In the case of the weaker reactions, the following scheme is employed. When the total number of pluses, let us say, is 7, the average being $2\frac{1}{3}$ plus, the reaction is 2 plus. If, however, the total number of pluses is 8, the average in this case being $2\frac{2}{3}$ plus, the reaction is 3 plus. Kahn recommends reporting the complete findings with the three tubes as well as the average.

INTERPRETATION OF RESULTS IN TEST WITH SERUM.

Serum : Reagent Dilution Reagent Dilution, c.c. Serum, c.c.		Tube 1 = 3:1 0.05 0.15	Tube 2 = 6:1 0.025 0.150	Tube 3 = 12:1 0.0125 0.1500	Average Results
Some typical reactions	1	++++	++++	++++	++++
	2	+	++++	++++	+++
	3	−	++	++++	++
	4	−	+	+++	+
	5	−	+	++	+
	6	−	−	−	−

Negative Reactions.—The appearance of negative reactions is opalescent and faintly milky. When serum alone is diluted with salt solution it appears opalescent and sharply clear. This difference in appearance between diluted serum and diluted precipitation reagent; also precipitation reagent suspension serum-mixture renders it easy to detect certain errors in technique such as omission of the addition of the precipitation reagent suspension, etc.

Interpretation of the Serological Results in Accordance with the League of Nations Committee.[1]—The nomenclature adopted by this committee corresponds to that of Citron's for the Wassermann tests: Kahn reaction.

++++, +++ = positive
++ = doubtful
+, ± = doubtful
− = negative

Specificity and Sensitiveness of the Test.—A positive reaction with this test, properly controlled, is claimed by Kahn to have the same diagnostic value as the result of a reliable Wassermann test. He tested over 500,000 serums by this method and found the results highly specific for syphilis. Also at the League of Nations Health Committee Conference (1928) 957 serums were examined by the Kahn method and compared with thirteen other methods. The Kahn method gave no false positive results. As is true with the Wassermann test, weak reactions should not be considered as diagnostic of syphilis. Leprosy, yaws and other conditions in which the Wassermann occasionally gives a false reaction are yet to be studied with the Kahn test. With regard to sensitiveness, this test may be considered as approaching that of a cholesterinized fixing reagent Wassermann test, but claimed to be more specific on the basis of the elimination of the hemolytic system.

The Quantitative Test with Serum.—The quantitative procedure enables one to determine the number of reacting substances in syphilitic serum. To differentiate this method from the routine test with serum the results are expressed in units rather than plus signs.

The Unit Reaction.—When 0.15 c.c. undiluted serum gives complete precipitation with 0.0125 c.c. of standard precipitation reagent dilution, the result is interpreted as a 4-unit reaction. By using a series of serum dilutions, the relative potency of any serum may be quantitatively determined on the basis of the formula S = 4 D, where S is the potency expressed in units and D the maximum dilution giving complete precipitation. Thus, if 1 to 5 represents the highest dilution of serum capable of giving complete precipitation, this serum contains 4 x 5 or 20 reacting units. If 1 to 50 represents the highest dilution in which a serum gives a complete reaction, the serum contains 4 x 50 or 200 units.

Procedure.—A serum is diluted with salt solution in series from 1 to 1 to 1 to 60. The dilutions may be prepared as follows:

1:1	undiluted serum.	
1:5	0.2 c.c. undiluted serum	+ 0.8 c.c. salt solution.
1:10	0.6 c.c. 1:5 dilution	+ 0.6 c.c. salt solution.
1:20	0.2 c.c. 1:10	+ 0.2 c.c. salt solution.
1:30	0.2 c.c. 1:10	+ 0.4 c.c. salt solution.
1:40	0.1 c.c. 1:10	+ 0.3 c.c. salt solution.
1:50	0.1 c.c. 1:10	+ 0.4 c.c. salt solution.
1:60	0.1 c.c. 1:10	+ 0.5 c.c. salt solution.

Eight tubes are set up and 0.01 c.c. standard precipitation reagent dilution pipetted into each in the usual manner. The serum dilutions in 0.15 c.c. quantities are now added, in order, to the tubes containing precipitation reagent dilution. The rack is shaken for three minutes, 0.5 salt solution added to each tube, and the results read and recorded. If a fifteen-minute incubation period at 37° C. is employed, it should be carried out as suggested in connection with the regular test, namely, after the shaking period and prior to the addition of salt solution.

[1] Copenhagen, May 21 to June 4, 1928.

COMPARATIVE POTENCY IN REACTING UNITS OF EIGHT SERUMS AS INDICATED BY TESTS EMPLOYING VARYING SERUM DILUTIONS WITH A CONSTANT AMOUNT OF PRECIPITATION REAGENT DILUTION.

Serum : salt solution Serum dilution, c.c. Precipitation reagent dilution, c.c.	1:1 0.15 0.01	1:5 0.15 0.01	1:10 0.15 0.01	1:20 0.15 0.01	1:30 0.15 0.01	1:40 0.15 0.01	1:50 0.15 0.01	1:60 0.15 0.01	Number of reacting units.†
Serum No.									
1	++++*	−	−	−	−	−	−	−	4
2	++++	++++	−	−	−	−	−	−	20
3	++++	++++	++++	−	−	−	−	−	40
4	++++	++++	++++	++++	−	−	−	−	80
5	++++	++++	++++	++++	++++	−	−	−	120
6	++++	++++	++++	++++	++++	++++	−	−	160
7	++++	++++	++++	++++	++++	++++	++++	−	200
8	++++	++++	++++	++++	++++	++++	++++	++++	240

* ++++ Complete precipitation reaction.

† Obtained by formula S = 4 D where S = potency of serum expressed in units and D the maximum dilution of serum giving complete precipitation.

Interpretation of Results of Quantitative Test.—Only complete or practically complete precipitation reactions are read in this procedure. Weaker reactions are considered negative. The procedure furthermore is applicable only to strongly positive serums, since only such serums would give reactions after dilution. A routine test, therefore, is always made before employing the quantitative test with a given serum.

Value of Quantitative Procedure.—The quantitative procedure is believed to be of especial value in following the serological effect of treatment. While the routine test may continue to give 4 plus reactions with the serum of a patient under treatment, the quantitative procedure indicates the actual change in the relative number of reacting substances.

The Test with Spinal Fluids.—When spinal fluids are used with antigen in the same proportion as serum, or in twice the serum amounts, the test may not be sufficiently sensitive when the spinal fluid is poor in "reagin." It is necessary to concentrate the reacting substances in the spinal fluid. This is done by precipitating the globulins with ammonium sulphate, centrifugalizing and taking up the precipitate in a small amount of salt solution. This method of concentration (suggested by Herrold) increases the sensitiveness of the test to a considerable degree.

Titration of Precipitation Reagent.—Reagent dilution shows a tendency for precipitation with solutions of ammonium sulphate. It is necessary, therefore, to carry out a special titration of the reagent for use in the spinal fluid test. It will usually be found that, if the titre of a given reagent for the test with serum is 1 + 1 with salt solution, the titre of the same reagent for the test with spinal fluid will be 1 + 1.3 or 1 + 1.4 with salt solution.

This special titration differs from the method for titrating reagent for the test with serum in that the solubility of the precipitates in the reagent dilutions is tested with a 3 per cent. solution of ammonium sulphate in physiological salt solution. This is necessary because the concentrated spinal fluid to be tested for specific reacting substances contains approximately 1 to 2 per cent. ammonium sulphate.

Five 1 c.c. quantities of cholesterinized reagent are measured into five standard reagent dilution tubes. Into five similar tubes are measured 1.1, 1.2, 1.3, 1.4 and 1.5 c.c. physiological salt solution, respectively. Each of the saline amounts is poured into a reagent tube and the mixture, in each case, immediately poured back and forth six times. These five reagent-saline dilutions contain precipitates and, after thirty minutes' standing, the solubility of these precipitates is tested with an ammonium sulphate solution prepared by mixing 3 c.c. of saturated solution with 9.7 c.c. normal saline. These tests are carried out in duplication employing 0.01 c.c. amounts of the five reagent dilutions with 0.15 c.c. of the salt solution containing 3 per cent of saturated ammonium sulphate solution.

The proportion of the above diluent to the reagent dilutions is 15 to 1. The reagent dilutions are pipetted to the bottom of the tubes with a 0.2 c.c. pipette graduated in 0.001 c.c. The ammonium sulphate solution is then added, the tubes shaken for three minutes and 0.5 c.c. saline added to each tube to render easier the reading of the results. The dilution of reagent and saline giving a precipitate which is soluble in the ammonium sulphate solution represents the titre of the reagent for spinal fluids.

Procedure of Test with Spinal Fluid.—Preliminary to the test, the spinal fluid is centrifuged to remove all cellular material. Two cubic centimeters of the clear fluid are mixed with 2 c.c. of saturated solution of chemically pure ammonium sulphate.[1] Incubate in a water-bath at 56° C. for fifteen minutes to hasten precipitation. Centrifuge at high speed for ten minutes to completely precipitate the globulin. Remove the supernatant fluid as completely as possible by means of a capillary pipette, add 0.15 c.c. of salt solution to the precipitate and redissolve it by gentle shaking taking care not to wash off the ammonium sulphate on the sides of the tube. This concentrated globulin solution is tested in essentially the same manner as serum in the qualitative test.

[1] Merck's Blue Label ammonium sulphate gives good results.

TYPICAL PRECIPITATION REAGENT TITRATION FOR USE WITH SPINAL FLUID.

Reagent dilution series Reagent + salt solution c.c.	1 1 + 1.1	2 1 + 1.2	3 1 + 1.3	4 1 + 1.4	5 1 + 1.5
Result of dilution	Heavy precipitate in each precipitation reagent dilution				
Scheme used in testing Solubility of precipitate in each reagent dilution	Tube No.			1	2
	*Reagent dilution c.c.			0.01	0.01
	3 per cent. solution ammonium sulphate in physiological salt solution c.c.			0.15	0.15
	Tubes are shaken three minutes and 0.5 c.c. salt solution added to each. All are observed for precipitates.				
Solubility of precipitate	Precipitate not soluble	Precipitate not soluble	Precipitate not soluble	Precipitate soluble	Precipitate soluble
				Reagent + minimum amount of normal saline giving precipitate which dissolves in 10 per cent. ammonium sulphate solution	

* Each reagent dilution is allowed to stand 30 minutes after mixing reagent and salt solution before solubility test is made.

Qualitative Test of Spinal Fluids.—Mix salt solution with precipitation reagent in the same manner as for the test with serum, according to the required reagent titre for spinal fluids. Allow the reagent to stand ten minutes at room temperature.

To each of two tubes is added 0.01 c.c. reagent dilution by means of a 0.2 c.c. pipette. Concentrated spinal fluid in 0.15 c.c. amounts is now added to each, also with a 0.2 c.c. pipette, and mixed with the reagent dilution. The tests are shaken for three minutes, after which 0.5 c.c. salt solution is added to each tube and the reactions read as in the test with serum. The reactions should be the same in both tubes. A clear-cut precipitate is read 4 plus. Precipitates of proportionally lesser intensity are read 3, 2 or 1 plus.

The controls for any series of spinal fluid tests include a positive and negative fluid concentrated by means of ammonium sulphate as in the case of the regular test; a reagent dilution control as described in connection with the serum test; a concentrated fluid control to establish that it is free from particles of any kind. The following special ammonium sulphate saline control is essential. Three c.c. salt solution are measured into a tube and 3 c.c. ammonium sulphate added. After thorough mixing, the tube is emptied, in the same manner as the spinal fluid tube is emptied after centrifugalization, and 0.3 c.c. salt solution added. A regular test performed with 0.15 c.c. of this control ammonium sulphate solution and 0.01 c.c. standard reagent dilution showed no precipitation.

Status of the Test with Spinal Fluids.—The test with spinal fluids is claimed by Kahn to approach the Wassermann test in specificity and sensitiveness.

Kahn Precipitation Test with Chancre Fluid.—The fluid is collected and clarified by centrifugalization. It is used without inactivating. A small amount of the fluid is added to about one-tenth its volume of standard reagent suspension and the mixture shaken for three minutes. A small amount of saline is added to

the mixture and the results read. A ++++ and +++ is read positive; ++ or + is read weakly positive, and ± or negative is read as negative.

Miscellaneous other fluids from syphilitic patients yielded negative with this test.

A Microscopic Slide Precipitation Test for Syphilis.—Kline and Young[1] have devised a modification of the Kahn test for which they claim the following advantages:

1. The test may be carried out on a glass slide instead of in a test-tube.
2. It is independent of variations in room temperature.
3. The reagent dilution used for the test will keep for several days.
4. It requires much less serum.

The reagent is an acetone-insoluble alcoholic extract of dried beef heart, plus cholesterin.

The glass slide used in the test is the ordinary 2 by 3 inch microscope slide upon which are placed twelve paraffin rings. The rings are made by a wire loop 13 mm. in diameter which is dipped into melted paraffin and applied to the slide.

With a pipette graduated in 0.01 c.c. place 0.05 c.c. of each serum to be tested in separate rings. The serum is previously heated to 56° C. for thirty minutes.

With a pipette which will deliver drops of approximately 0.0075 c.c., 1 drop of reagent is allowed to fall into each serum. The slide is then rotated with a tipping motion so that the mixture of serum and reagent will take place most intimately. This is continued for three and a half minutes, rotating the slide once each second.

The readings are made through a microscope (16 mm. obj., 10 or 12.5 eyepiece with the light cut down). The results are recorded in terms of pluses according to the degree of clumping.

After carrying out several thousand precipitation tests in comparison with Wassermann tests as done in the Laboratory of the Department of Health, we have found that a combination of these two gives an added security when applied to routine examinations for syphilis.

The Meinicke Clarification Reaction for Syphilis.[2]—This reaction differs from the other precipitation reactions in several particulars.

The patient's serum is used without being heated and hemolyzed or cloudy serum does not interfere with the reading.

The reagent is an alcoholic extract of dried beef-heart plus 2 per cent of balsam of Tolu. This is diluted with a sodium carbonate—sodium chloride solution and produces a white opaque solution having the general appearance of whole milk.

The results are easily read because the positive reactions are indicated by a perfectly clear watery solution while the negative reactions retain the densely opaque appearance of the original reagent.

Four dilutions of the reagent are used and the results are read after eighteen hours at room temperature. 0.2 c.c. of serum is required for each of the four tubes, and the total volume of the test is 0.7 c.c.

In addition to the four tube macroscopic reactions, Meinicke has a micro-reaction which is performed with one loopful of serum and one loopful reagent on a coverglass and a hollow ground slide.

This reaction is complete in one hour and is read with the aid of a microscope, using a low power. The positive reaction is indicated by a distinct agglutination while the negative remains finely granular.

[1] Kline, B. S., and Young, A. M.: Jour. Am. Med. Assn., 1926, **86**, 928; Jour. Lab. and Clin. Med., 1927, **12**, 477. Klein, B. S.: Jour. Lab. and Clin. Med., 1928, **13**, 588.

[2] Münch. Med. Woch., 1929, No. 8, p. 318.

CHAPTER XIII.

OPSONINS AND PHAGOCYTOSIS.

ANTIBACTERIAL SERUMS FOR THERAPEUTIC USE. ACTIVE IMMUNIZATION VACCINES.

Although it had been suggested, by earlier observers, that ingestion of microbes by the body cells was a means of destroying the infecting agent, Metchnikoff was the first experimentally to prove this fact. This ingestion is followed by a digestion and is analogous to the feeding processes of unicellular organisms. Metchnikoff noticed that phagocytosis was more active in the presence of immune serum than with normal serum, but thought that this was due to the presence of leukocyte-stimulating substances in the serum. Denys and Leclef suggested

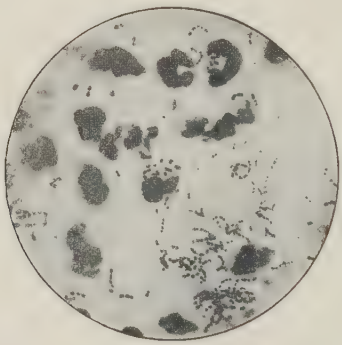


Fig. 91.—Streptococcus engulfed by leukocytes from abscess. Gentian-violet stain. × 1000. (Fränkel and Pfeiffer.)

that the action of immune serum might be upon the microbes reducing their resistance of phagocytosis. By utilizing the technic of Leishman, Wright and Douglas definitely proved the existence of a substance in serum which, acting upon the microbe, prepared it for phagocytosis. This substance Wright gave the name "opsonin." Neufeld applied the term "bacteriotropin."

The Phagocytic Cells.—Phagocytic capacity is possessed by two general types of cells, the leukocytes of the blood and the cells of the reticulo-endothelial system. The latter comprises the endothelial cells of the spleen, bone-marrow and lymph nodes, the Kupfer cells of the liver, the histocytes or clasmatocytes of connective tissue and the monocytes of the circulating blood. Metchnikoff used the terms microphage and macrophage to designate the two general types of phagocytic

cells. In general, there are two forms of inflammation, depending on the attraction or non-attraction of leukocytes. If a subcutaneous infection occurs, due to the streptococcus, there is an inflammatory response which is dominated by the collection of polymorphonuclear leukocytes which are attracted ("positive chemotaxis") and pass through the wall of the capillaries to the tissues. At the same time there is an accumulation of serous exudate. The polymorphonuclear leukocytes as well as the other types of phagocytic cells attempt to dispose of the bacteria. If the infection progresses the accumulation of cells continues and the leukocytes and tissue cells which are killed form the resulting "pus." This consists mostly of the accumulated leukocytes and serous elements.

If the infection is due to tubercle bacilli a different type of inflammation occurs. The bacilli are rapidly surrounded by large mononuclear cells, apparently endothelial in origin, and about the latter will develop an exudation of cells of the lymphocyte type. The polymorphonuclear variety is not attracted ("negative chemotaxis"). Phagocytosis by the endothelioid cells and the giant cells formed from these is noticed. If the infection progresses, necrosis develops in the center of the cell mass, resulting in caseation. The polymorphonuclear leukocytes, however, play a rôle in the preliminary attempt to dispose of the bacilli, as is seen in the phagocytosis when tubercle bacilli are introduced into a serous cavity.

In general infections the blood picture—total leukocyte and differential count—gives evidence of the positive or negative chemotactic character of the infecting organism. If an infection is due to an excessively virulent organism or if infection be extensive and severe a negative chemotaxis results even though the infecting agent is ordinarily of the positive variety.

Buxton and Torrey[1] emphasized the macrophage as an important factor in the recovery from or the immunity to infection by bacteria introduced intraperitoneally. Gay[2] recently has studied the activity of these cells in pleural and other forms of local streptococcus infection. He presents evidence that the macrophage or clasmatocyte is the essential agent in the recovery from local infection by streptococci and that there develops a local immunity after such infection for which the clasmatocytes are responsible. The preliminary production of a pleural exudate of polymorphonuclear leukocytes resulted in no increased local resistance, whereas the preliminary production of an exudate of clasmatocytes led to protection. However, this local resistance is apparently not specific, as the mobilization of clasmatocytes may be induced by non-specific substances, such as gum arabic broth. The clasmatocytes of one pleural cavity may migrate to the other. The mobilized clasmatocytes disappear in about two weeks. Other observers had noted the association of macrophages with streptococcus infection.

[1] Jour. Med. Res., 1906, **10**, 3; 1907, **11**, 251.

[2] Gay and Clark: Arch. Path. and Lab. Med., 1926, **1**, 847; Jour. Inf. Dis., 1925, **36**, 233; Proc. Soc. Exp. Biol., 1926, **24**, 20, and 1926, **24**, 23.

Opsonins.—Opsonins are present in normal serum and in greater amounts in the serum of immune animals. Evidence has been advanced that the "normal" and the "immune" opsonins are not similar substances.

The normal opsonins are nearly destroyed by heating to 60° C. for fifteen minutes. The action of normal opsonins is apparently due to a substance thermostabile at 55° C., the activity of which is very much enhanced by another serum element thermolabile at 55° C. and nonspecific in character. The latter substance is apparently the alexin or complement. The thermostabile substance is relatively specific in the sense that the absorption of normal serum by a specific organism will only remove those for itself or related organism, but not necessarily all the opsonic substances.

The immune opsonins or bacteriotropins (Neufeld) would resemble antibodies of the so-called second order (Ehrlich) if the evidence advanced was true as to their ability to withstand heating to 55° to 60° C. for forty-five minutes as well as their inability to be reactivated by the addition of normal serum. Dean, Hektoen and others, although admitting the thermostabile character of the immune opsonin, have shown that their action is enhanced by the addition of fresh normal serum, inactive in itself. It would seem, therefore, that there is no fundamental difference between the normal and immune opsonins or bacteriotropins. The apparent greater resistance of the latter may be due only to concentration, as the greater resistance to heat was found by Wright to disappear on dilution. Opsonic action is not completely lost on heating, thus apparently differing from the complete inactivation of bactericidal action brought about by the same means. This difference may only be apparent because the leukocytes used in the test may supply traces of complement which reactivates the opsonic antibody. This hypothesis, however, rests on the still doubtful contention that the leukocytes are a source of complement. The fact that the opsonin value and the bactericidal action of a serum do not necessarily parallel each other quantitatively, has been advanced as evidence that the opsonic action is due to a distinct antibody and not due to the action of bactericidal amboceptor either alone or with complement.

Immune opsonins or bacteriotropins like other antibodies are specific for the stimulating microörganism. Some group action, however, may be evident, with closely related types. As with other antibodies, microörganisms exhibit a variable degree of resistance to the action of opsonins. This again is relatively proportionate to their virulence and source, and can be artificially modified. The resistance to phagocytosis may not be wholly due to resistance to the combination of opsonins. Microörganisms may secrete substances which repel or actually injure the leukocytes (leucocidins). As has been previously noted, capsule production is apparently a protection against antibody action. Pathogenic bacteria which have been cultivated on artificial media for some time may be spontaneously phagocyted, that is, phagocyted in the absence of serum action.

Variations in Activity of Phagocytic Cells.—According to the facts given above, one would conclude that the increased phagocytic capacity of the cells of the immune host was wholly due to the increased opsonins. It has been shown that in lobar pneumonia the leukocytes may show an increased phagocytic power without regard to serum action. This may be due to other factors, as for instance, the age of the cell, rather than to an acquired cell characteristic.[1] Park and Biggs have shown that differences exist between the cells of apparently healthy individuals. Probably an equal degree of difference occurs with cells of the same individual at different times.

Mechanism of Phagocytosis.—The phenomenon of chemotaxis and of phagocytosis can be simulated with inanimate agents. A study of these agents indicates that chemotactic action is due to substances which lower the surface tension of the cell. This causes attraction and results in phagocytosis, thus if the leukocyte meets a substance which reduces its surface tension it flows about or engulfs the substance. After ingestion, microörganisms are subjected to the action of the endolysins or ferments by which they are destroyed and digested.

Endolysins and Endo-enzymes.—Leukocytes, as shown by Schattenfroh, contain bactericidal substances. These can be extracted. They differ from similar serum antibodies in that they are more thermostabile, a temperature of 75° to 80° C. being necessary to destroy them. These substances have been termed "endolysins." Zinsser showed that one could extract no greater amount of these substances from the cells of immune than from normal individuals. Endolysins are not specific. Little if any of this substance is contained in the lymphocytes and macrophages. A number of enzymes have also been obtained from phagocytic cells. Opie obtained two proteolytic ferments from the cells of exudates, one from the polymorphonuclear cells, active in weak alkaline solutions, the other from exudates containing large numbers of mononuclear cells, active in weak acid solutions. Because of the presence of the above substances, extracts of leukocytes may on injection have protective or therapeutic value. The leukocytes possess no lipase which, however, is present in the macrophages. The leukocyte, therefore, cannot digest acid-fast bacilli such as the tubercle bacillus. Tubercle bacilli or other microörganisms which resist the power of the leukocytic endolysins and endoferments may be disseminated by what is essentially a protective mechanism, the wandering phagocyte.

Technic of Demonstration and Measurement of Opsonic Action.—Viable leukocytes may be obtained from sterile exudates or from the blood. In the former a 5 per cent. aleuronat suspension in a 3 per cent. starch solution in broth or a 25 per cent. solution of peptone is injected intraperitoneally into a guinea-pig or intrapleurally into a rabbit. After sixteen to twenty-four hours the animal is killed and the exudate

[1] Potter and Krumwiede: Jour. Inf. Dis., 1907, **4**, 601.

is collected and added to about 20 c.c. of a 1 per cent. sodium citrate in an 0.8 per cent. salt solution. This is centrifugalized, the leukocytes are again suspended in saline and are again sedimented three or four times successively to wash away traces of serum. The leukocytes are then suspended in saline. Leukocytes are obtained from the blood by adding 1 part of blood to 15 to 20 parts of citrate salt solution, which prevents clotting. Small amounts, 1 c.c., may be obtained by deep puncture of the finger or the blood may be obtained from the vein by syringe. The blood of experimental animals may be used. The diluted blood is centrifugalized at low speed. The red cells having the greater specific gravity are sedimented first, the leukocytes last, collecting as a creamy layer over the red cells.

The supernatant fluid is removed without disturbing this layer, and the leukocytes are collected with a capillary tube. They will be mixed with red cells. The leukocytes are now placed in saline in another centrifuge tube, centrifugalized and the cells washed free of serum as described above, and finally suspended in saline. The solutions employed should be warmed to 37° C., as cold will affect the activity of the cells. Centrifugalized at too high speed is to be avoided, as this may clump the leukocytes. For careful work, and especially if the activity of different cells is to be compared, the cells in the suspensions should be counted and standardized to a definite content of polymorphonuclear leukocytes.

The bacterial suspension may be either a saline suspension of the growth on agar or a broth culture. The culture selected must not give spontaneous phagocytosis nor should it be unduly resistant to opsonic action. The suspension must be of a satisfactory density.

The serum is collected from man by puncture, allowing the blood to flow into a Wright capsule. The blood of experimental animals is collected from the vein and placed in a test-tube to clot. If necessary the clot is loosened from the glass so that it will contract and the serum separate.

The Method of Wright-Opsonic Index.—This method gives a comparison between the opsonic action of a serum, to be tested, and that from a normal individual or the pooled serum from several normals. The latter constitutes the control or standard measure. A capillary pipette is made and marked about an inch from the end. With a rubber teat the leukocyte suspension is sucked up to this mark, then a small bubble is allowed to enter the tip, then the bacterial suspension is sucked up to the mark, a bubble allowed to enter and last the serum drawn up to the mark. We now have three equal quantities of cells, bacteria and serum, separated by bubbles. The contents are then mixed by blowing out the contents on a slide and sucking it up, repeating this several times. Bubbling must be avoided. The contents are sucked well up into the tube, the tip is sealed in the flame and the tube incubated at 37° C. for twenty minutes or longer. The tip is then nicked with a file and the contents again mixed, a drop placed on a slide, a smear prepared, using another slide, or cigarette paper as a spreader. This is then fixed and stained

and examined with the oil-immersion lens. The leukocytes are more numerous along the edges of the smears. The average number of bacteria per leukocyte is determined by counting the contents of 50 or preferably 100 cells. This is done with the normal or control serum and

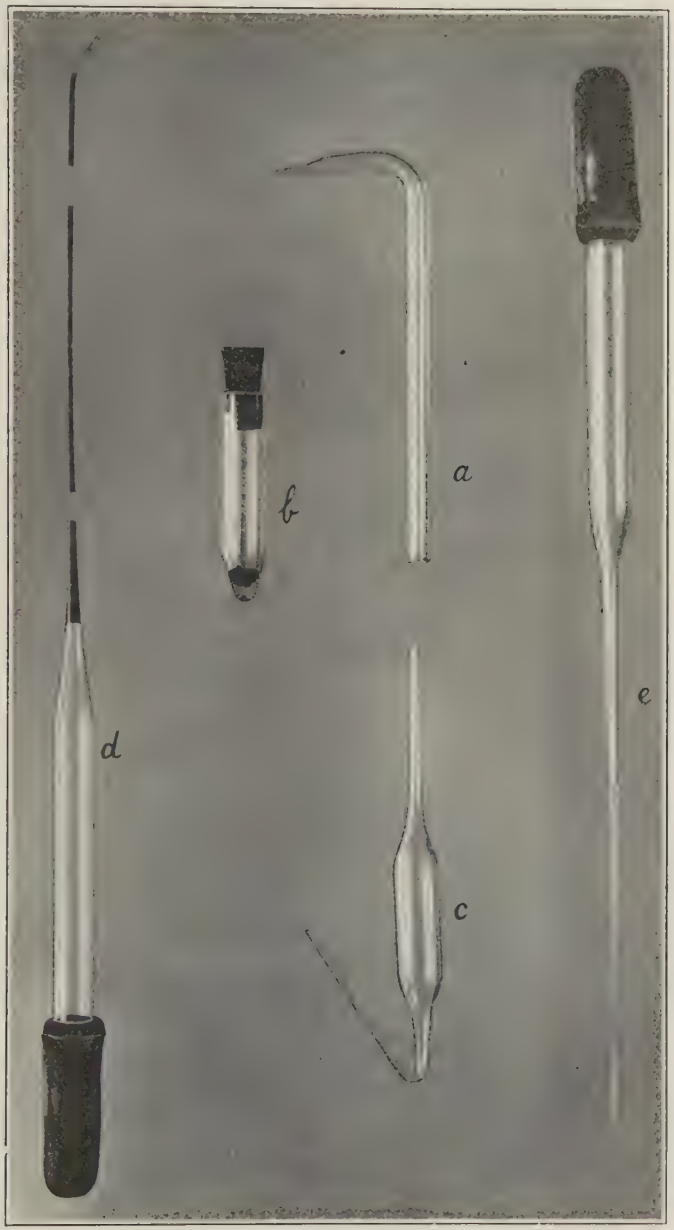


Fig. 92 —Opsonic outfit. (Wright)

with the serum for test. The result is expressed by the opsonic index thus:

$$\frac{\text{5 per leukocyte-test serum.}}{\text{10 per leukocyte-control serum.}} = \text{Opsonic index; 0.5.}$$

Before carrying out this method the bacterial suspension must be standardized so that not more than 5 to 10 bacteria per leukocyte will be taken up in the time of incubation. This is done by preliminary tests with the suspension and dilutions of the suspension.

Method of Neufeld.—This method involves the determination of the highest dilution that still causes phagocytosis. Dilutions of the serum to be tested as well as of a normal serum (same species) for control are prepared. Mixtures may then be made as with the Wright technic, or 0.1 c.c. of the bacterial suspensions and serum dilutions may be mixed, incubated to sensitize the bacteria, 0.1 c.c. of the leukocyte suspension then added and the mixtures again incubated for twenty to thirty minutes. Smears are then made from each of the tubes. A salt solution control should also be included. Neufeld advises the inactivation of immune sera.

Accuracy of Opsonic Determinations.—In one and the same smear marked differences will be noted in the degree of phagocytosis of different leukocytes. To arrive at any degree of accuracy, therefore, with the Wright method a considerable number of cells must be counted. This is clearly shown in the following table:

OPSONIC INDEX ESTIMATIONS IN FIVE BLOOD SPECIMENS.

Cells counted.	Average number of bacteria in each leukocyte.				
50	1.18	1.88	1.34	1.42	1.90
100	1.22	1.78	1.24	1.42	1.59
150	1.18	1.62	1.22	1.44	1.50
200	1.18	1.51	1.22	1.46	1.37
600	1.28	1.62	1.23	1.36	1.36
1200	1.34	1.44	1.25	1.30	1.42

One difficulty with the Wright method is the selection of a normal control. We now know that many healthy individuals appear to have been actively immunized to many varieties of bacteria. This is probably due to slight infections or merely to being germ carriers. Even the use of pooled serum does not exclude some variation. The degree of such variation is evident from the following:

OPSONIC COUNTS IN TEST OF TWENTY-ONE NORMAL SERA WITH STOCK STAPHYLOCOCCUS CULTURE.

1	4.13	8	3.82	15	9.09
2	2.93	9	3.95	16	5.17
3	2.78	10	3.98	17	4.04
4	4.37	11	4.27	18	3.82
5	3.58	12	3.69	19	4.00
6	2.90	13	3.80	20	3.79
7	3.56	14	3.59	21	3.44

The Wright and the Neufeld methods are likely to give extremely discordant results because of actual differences among the leukocytes, the number in the suspension and the culture employed. The latter will vary according to the strain employed and differences in density of suspension will also lead to variable results. Individual observers will estimate or count slightly differently which will be a further factor. These factors become very evident when sera are submitted to four or five competent observers. Under these conditions variations of 10 to 20 per cent. are common and at times differences of 100 per cent. are encountered. In determining the opsonic content of therapeutic sera obtained from the horse, the high content of normal opsonins is a further factor of error. On the whole the method has many variants which are difficult to control.

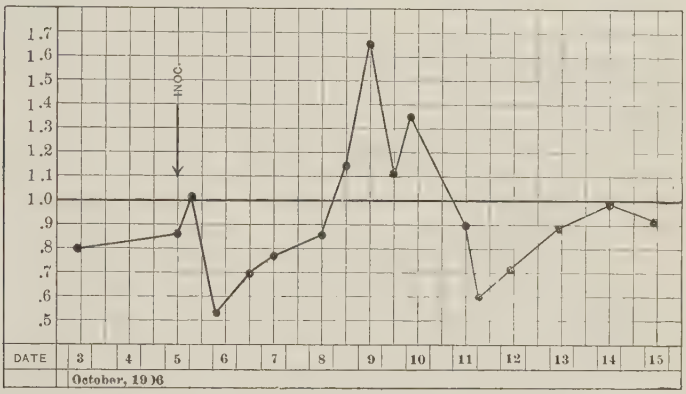


FIG. 93.—Opsonic curve, showing the slight immediate rise and the later negative and positive phases following inoculation. The changes here are more regular than generally occurs.

Applications of the Opsonic Determinations.—Wright advocated the method as a means of control of vaccine therapy. Where vaccines are injected, Wright states, there "supervenes a negative phase where there is a diminished content in protective substances. This is succeeded by a positive phase. This inflowing wave of protective substances rapidly flows out again, but leaves behind in the blood a more or less permanently increased content of protective substances. When a small dose of vaccine is given the negative phase may hardly appear, but the positive phase may be correspondingly diminished. Where an unduly large dose of vaccine is inoculated the negative phase is prolonged and much accentuated. The positive phase may in such a case make default. It will be obvious that if we, in the case of a patient who is already the subject of a bacterial invasion, produce by the injection of an excessive dose of vaccine a prolonged and well-marked negative phase, instead of benefiting the patient we may bring about conditions which will enable the bacteria to run riot in his system."

"Now consideration will show that we may obtain, according as we

choose our time and our dose wisely or unwisely, either a cumulative effect in the direction of a positive phase or a cumulative effect in the direction of a negative phase. We may in other cases, by the agency of two or more successive inoculations, raise the patient by successive steps to a higher level of immunity, or, as the case may be, bring down by successive steps to a lower level. We can select the appropriate time and dose with certainty only by examining the blood and measuring its content in protective substances in each case before reinoculating."

The reasons for the discontinuance of this method are several: (1) the relative inaccuracy of the method; (2) the fact that almost equal differences can be observed in normal or diseased individuals from day to day without regard to injections of vaccines (Fig. 94); (3) injections are based, not on the index at the time of injection, but of the previous day because of the exigencies of the opsonic method; (4) the development of a negative phase is apparent, not real; (5) equally satisfactory results are obtained, controlling dosage by the degree of focal and systemic reaction.

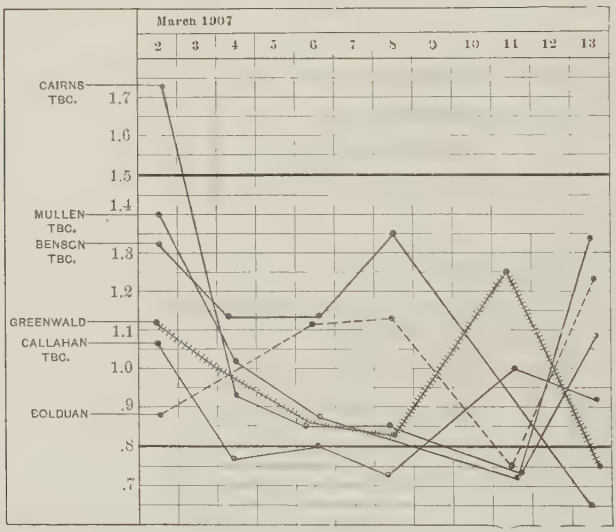


FIG. 94.—Dotted and crossed lines, normal persons. Continuous lines, tuberculosis.

The determination of the opsonic content of immune horse serum to be used for therapeutic purposes has been employed especially for the standardization of antimeningococcus serum. The method is far from satisfactory as a general routine test for the reasons given. The method is used to some extent as an additional control on other methods of standardization. It is also used as a means of grouping meningococci as regard their reactions to the opsonins of different serums and "opsonogenic" characteristics. For this work a modified Neufeld method is used.[1] The determination of the presence of an increased opsonic con-

[1] Evans: Jour. Immunol., 1922, **7**, 271.

tent has been utilized to a limited extent as a diagnostic procedure. On the whole, the method is only applicable to special investigative work and the results must be scrupulously controlled and the results verified by repetitions of the test.

Opsonins for Other than Microbal Cells.—As with other antibodies, these may be easily produced. Those for red cells, hemopsonins, have been mostly employed for experimental purposes in determining the factors of opsonic action.

Leukocytic Extracts.—Hiss and Zinsser prepared a leukocytic extract for therapeutic purposes. They injected rabbits intrapleurally with an aleuronat suspension, killed them after twenty-four hours, added the exudate to citrate solution and finally extracted the cellular sediment with distilled water.

Experimentally they showed that such extracts possessed protective value in infected rabbits. In many cases they, and others, obtained favorable therapeutic results in man in the treatment of pneumonia, staphylococcus infections, meningitis, influenza and other types of infection. Zinnser believes the use should be confined to local infections. The extract is given in 10 c.c. doses every four to six hours. Although beneficial results are apparent in some cases, other cases have shown no benefit. On the whole the results have not been striking. Large amounts of extract may be prepared by using horses.

Antibacterial Serums for Therapeutic Purposes.—Sera having a high antibody content also show considerable protective value on animal injection. Likewise, therapeutic value is indicated for such sera in that they prevent or delay the death of experimental animals even when the serum is administered after the infecting dose of bacteria is given. The relative effect, however, is directly proportionate to the shortness of time which elapses between infecting and serum dose. This is partly due to limitations of such animal experimentation. These are imposed by the fact that we are not reproducing the natural disease in the animals but causing an infection, usually septicemic, the only reliable criterion of the severity of which is the death or recovery of the animal. Because of these facts, antibacterial sera have been utilized in man for both prophylactic and therapeutic purposes.

General Methods Used in Production of Antibacterial Sera.—*Immunization of Horses.*—Subcutaneous injections have been practised in the past, but this method has certain objections which have led to the almost general adoption of intravenous inoculation. With the latter method, a high degree of immunity is more quickly established, the amount of culture required is less, and certain complications avoided, such as the development of abscesses after meningococcus vaccines. The frequency of inoculation will depend on conditions. Daily intravenous injections alternating with periods of rest is the most rapid method of producing antisera, giving 3 to 5 successive daily injections followed by several days to a week's rest. The Dochez agar method (see under Antitoxin) results in a good antibacterial response, but not as good or as quick a one as that obtained by intravenous injection of the cocci.

Preparation of Vaccine.—This varies with the bacterium employed. The easily growing types are cultivated on agar and suspended in saline. Meningo-

cocci are grown on glucose agar. Streptococci or pneumococci may be grown on agar or in broth. If the latter, the cocci are thrown down and then washed with saline solution and finally suspended in saline for injection. Injection of the broth of culture ingredients is to be avoided, as serious reactions or even sudden death may occur when large amounts are given. This has been attributed to the peptone-like substances. With meningococci, because of the toxicity of autolytic products, as little delay as possible should occur between preparation of the suspension and its injection. In the case of meningococci live organisms are usually used. With pneumococci, or other varieties which may infect the horse, it is more desirable to use killed bacteria. Beard is using 0.3 per cent. formalin in the suspensions. This also limits the autolysis of the meningococci. Stock strains are used for the other species of microörganisms.

Selection of Strains.—This will depend on whether a univalent, polyvalent or a serum against representative strains is desired. In the case of meningococci the strains should cover all agglutinative variants. The use of a few selected dominant cultures of the main Gordon groups apparently is all that is necessary. With pneumococci, a serum against several types can be produced with antibody content against each almost as active as in univalent serums, but as a rule the horse does not stand such injections for any considerable period. With streptococci, the representative hemolytic strains are employed as we have little reason to include the viridans type, since they are practically heterogeneous, and even sera which we have prepared with individual patient's strains have given no results in chronic endocarditis. Torrey's strains of gonococci are usually employed. For dysentery sera the representative types are employed. It is essential that the most dominant type of a group be employed and that the strains be retested from time to time to be sure that degradation is not taking place. (See under Agglutination.)

Horses.—Healthy horses are selected. (Deformity or crippling is no bar.) They are tested for glanders with mallein and by the complement-fixation reaction. An injection of tetanus antitoxin is given every few months.

Dosage.—The dosage depends on the toxicity of the strains employed and the temperature and general reaction of the horse as well as his general health. Specific dosage, therefore, cannot be quoted. Depending on the type of bacterium $\frac{1}{20}$ to $\frac{1}{10}$ of an agar slant or 1 to 2 c.c. of a broth culture is the initial intravenous dose. This is then gradually increased until a maximum is reached. When injections are given only once a week, larger doses may be given than when given daily. After the injections, temperatures should be taken frequently. The optimum dose causes a sharp febrile reaction to about 104° F., but the temperature returns to normal in eight to twelve hours. A chill may occur. The maximum dose is the amount that will maintain the required antibody content and the balance of antibodies if several strains are employed, but if this requires doses which give excessive reactions, the horses' period of productivity will be short.

Titration of Sera.—*Standards.*—The methods employed and the standards adopted vary to a great extent, as the problem involved is much more complex than with toxin-antitoxin methods. The methods possible of application are dilution titration of the antibodies, agglutinins, precipitins, opsonins and bactericidal content and the determination of the protective value when injected with cultures. The agglutinin content is chiefly of value with typhoid and dysentery, although the content in these antibodies may decline after prolonged inoculation without, so far as we know, a coincident fall of other antibodies. The agglutination reaction is of some value in determining the probable antibody balance where several or many types of strains are employed as with dysentery and meningococci, this being the guide as to the pro-rata amounts to be injected into the horses. The value of this method is naturally influenced by the tendency of the horse to produce common agglutinins. Opsonic determination is only of limited value. The bactericidal titration (*in vitro*) has been used very little. The complement-fixation reaction is of value in titrating meningococcus serum;

and a rough standard that not more than 0.002 c.c. shall be required to give complete fixation, using a mixed antigen, is employed in several laboratories. The protective value of meningococcus serum, using white mice (Hitchens and Robinson[1]) is difficult to determine because of the low virulence of most meningococcus cultures. For pneumococcus serum protective experiments with white mice offers the best method. (See below.) Protection experiments can also be applied to antistreptococcus serum.

On the whole the methods other than the protection test are far from satisfactory, titration of one antibody does not necessarily give us any information as to the content in other antibodies. Protection tests are satisfactory where the bacterium is truly septicemic for the test animal, less so where invasion is less marked (meningococci) and of least value where death is due essentially to "endotoxins." The agglutinin content seems to be the least valuable as an indication of therapeutic potency.

Bleeding.—Bleedings for therapeutic sera are made with trocar and rubber tube into 2-liter Erlenmeyer flasks having a large flat wire egg-beater to support the clot. After the flask is about half-full, it is tilted on the side where the wire is inserted. The flasks may be stood up after the clot is firm. The serum separating after twenty-four and forty-eight hours is drawn off with siphon. All operations must be aseptic.

Serum.—The collected serum is placed in a sterile vessel and tricresol added, drop by drop, with vigorous stirring to prevent precipitation, until 0.4 per cent. is added. Krumwiede and Banzhaf[2] find that the addition of a mixture of equal parts of ether and tricresol will eliminate the difficulties due to precipitation and allows one to add all the tricresol needed without resorting to slow fractional addition. In two to three weeks, after which further fibrin separation usually ceases, it is passed through paper-pulp filters and then through a Berkefeld filter to remove any contamination occurring at the time the tricresol was added. The tricresol will prevent any multiplication of the contaminants prior to filtration. Instead of tricresol one may add several cubic centimeters of chloroform per liter and shake, the excess settles out with any separated fibrin and is avoided when the serum is drawn up in the bottling apparatus. The sera must be kept in the refrigerator at all times and tested to determine its sterility before being issued.

Concentration and Separation of Antibodies.—The evidence we have shows that most if not all of the protective ("curative") antibodies precipitate with the globulin fraction. Until recently no general practical application has been made of this fact. It has been known for a long time that antibody when combined with its antigen can be dissociated to a greater or less extent by the use of appropriate extractives. As was noted under Precipitins some success has been attained with antipneumococcus serum by dissociation of the antibodies from precipitates. Huntoon[3] has developed a method of obtaining an "antibody solution" freed of nearly all serum elements. His method is briefly as follows:

Pneumococci are emulsified in salt solution and added to antipneumococcus serum. The antibodies combine with the added organisms. Every effort is made to avoid the development of precipitin reactions since this carries down globulins from the serum.

After combination has taken place the organisms with the attached antibody are centrifugalized out; re-emulsified in cold salt solution to remove the serum clinging to the organisms, again centrifugalized, again emulsified and recentri-

[1] Jour. Immunol., 1916, **1**, 345 and 355.
[2] Jour. Infect. Dis., 1921, **28**, 367.
[3] Jour. Immunol., 1921, **6**, 117; see also Hyg. Lab. Bull., 1923, **124**, 45.

fugalized. This removes practically all serum constituents with the exception of the antibody.

The washed organisms with attached antibody are now emulsified in physiological salt solution containing 0.25 per cent. sodium bicarbonate and the emulsion heated to 55° C. with constant agitation for thirty minutes.

The emulsion is now centrifugalized while hot and the supernatant fluid retained. This contains antibody. This solution is chilled to 5°C., recentrifugalized and finally filtered through filter candles.

The resulting product should be water clear, and when injected in 5 c.c. doses intraperitoneally into guinea-pigs should produce sensitiveness to horse serum in not more than 50 per cent. of the animals so treated.

His conclusions in relation to the nature of antibodies are as follows:

1. The antibody is of a colloidal nature although under conditions a small amount will pass a celloidin membrane.
2. Antibodies in such solutions are not affected by digestion with trypsin, neither are they completely precipitated by saturated sodium chloride, by 80 per cent. saturation with ammonium sulphate, or by distilled water.
3. Antibodies are therefore not of the nature of euglobulin, or pseudoglobulin.
4. Antibodies are not destroyed by absolute alcohol.
5. Antibodies, although a serum constituent, are not of the nature of serum proteins as generally understood.

Felton[1] has developed a simple concentration method. It is based on the fact that the globulins of the serum are precipitated by the addition of distilled water. He has modified his original procedure, employing sodium sulphate for preliminary precipitation of the globulins, a procedure published by Banzhaf.[2] The globulin precipitate is collected and dialyzed until free of sodium sulphate. The dialysate is slightly acidified and filtered. The filtrate is diluted with 5 volumes of distilled water and the resulting precipitate collected. This is dissolved in saline, a preservative added and the solution filtered. A five- to ten-fold concentration of antibody is obtained.

Technic of Protection Test.—The culture used should be highly virulent or at least as virulent as possible, for the test animal (mouse, guinea-pig or rabbit). If not of high virulence, one should attempt to raise its virulence by animal passage. The minimal lethal dose of the culture is then determined using either the intravenous or intraperitoneal route. The actual test is then carried out by mixing an arbitrary volume of serum with increasing amounts of culture or an arbitrary amount of culture with decreasing amounts of serum. The mixtures are injected intraperitoneally (mice or guinea-pigs) or intravenously (rabbits) and the animals kept under observation. The following tables are examples of the titration of an antipneumococcus serum to determine its value in protective units. The procedure given is that of Cooper, which is similar to that of Felton.

PNEUMOCOCCUS PROTECTION TEST. CONSTANT AMOUNT OF SERUM.

Mouse.	Serum. c.c.	Broth culture. c.c.	Result.
1	0.2	0.05	Survived.
2	0.2	0.10	Survived.
3	0.2	0.20	Survived.
4	0.2	0.30	Dead 48 hours.

[1] Boston Med. and Surg. Jour., May, 1924.
[2] Proc. Soc. Exp. Biol., 1925, **22**, 329.

PNEUMOCOCCUS PROTECTION TEST. CONSTANT AMOUNT OF CULTURE.

Mouse.	Serum. c.c.	Broth culture. c.c.	Result.
1	0.001	0.001	Survived.
2	0.0005	0.001	Survived.
3	0.0002	0.001	Dead 72 hours.
4	0.000125	0.001	Dead 48 hours.
5	0.0001	0.001	Dead 72 hours.

Mouse	Colony count.	Broth culture. c.c.	Result.
Control A	223	0.000001	Dead 48 hours.
Control B	21	0.0000001	Dead 48 hours.
Control C	2	0.00000001	Dead 48 hours.
Control D	0	0.000000001	Survived.

The definition of a unit of antipneumococcus serum which is gaining acceptance is as follows: A unit is ten times the smallest amount of antiserum which will protect mice inoculated intraperitoneally with 100,000 fatal doses of a fully virulent culture.[1] In the example given above 0.00000001 c.c. of culture is a fatal dose; 0.001 c.c. contains 100,000 fatal doses and 0.0005 c.c. antiserum protects against this amount of culture and, therefore, contains 0.1 unit. Therefore, the antiserum has 200 protective units per cubic centimeter.

The minimum quantity of antiserum necessary to bring about the recovery of highly susceptible animals from infections, introduced into an organ such as the lung or into a tissue such as the skin[2] may be determined.

If agar cultures are employed (meningococcus) the fractions of the surface growth are the measure employed, or better, the number in suspension may be determined by actual count.

Every animal that dies must be cultured to exclude spontaneous infection due to its own septicemic types. If the test organism is invasive its presence can be demonstrated by smear or culture from the heart's blood. Further identification will be necessary when the injected type and the own septicemia type are similar.

We have stated that the protection test gives its best results when the bacterium is highly virulent.

It is probable that even with extremely virulent bacteria that multiple proportions hold between serum and culture only when amounts are used within a limited range which must be found by experimentation.

The average of some experiments of Dochez on pneumococcus protection show this, thus 0.1 c.c. of serum would protect against 0.5 c.c. of culture, 0.25 c.c. against 0.8 c.c., but 3 c.c. was necessary to protect against 1 c.c. We have found that when very small amounts of culture, such as 0.00001 c.c., 0.000001 c.c. and 0.0000001 c.c., are used that disproportionately large amounts of antiserum are required to protect. There is probably a minimum protective serum dose dependent on the

[1] 100,000 fatal doses of culture was chosen as the test dose rather than larger amounts because of an inhibition of protection phenomenon which is often found when large amounts of serum are titrated with a large dose of culture.

[2] Goodner: Jour. Exp. Med., 1928, **48**, 413.

size and susceptibility of the animal employed. This is in accord with the results of Neufeld and Handel. With moderately virulent cultures the limits may be still narrower, so that experimental errors in direct as well as in cross-protection tests to determine the relationships of cultures one to the other are easily made.

Cross-protection and Strain Identity.—The error of assuming strain identity on the basis of insufficiently controlled protection tests is not infrequent. Thus, if antiserum A will protect against Strains A, B, C, etc., this is advanced as proof of identity. As a matter of fact, it is only an indication of group relationships. The following schematic presentation of the results of Avery will show this very clearly:

PNEUMOCOCCUS PROTECTION TESTS.

Variety of anti-serum.	Variety of culture. Type II.	Subgroup IIa.	Subgroup IIb.
Type II	Protects	Protects	Protects
Subgroup IIa	No protection	Protects	No protection
Subgroup IIb	No protection	No protection	Protects

In other words the reverse phenomenon must be observed before conclusions are drawn. The question arises: would even mutual protection be complete evidence of identity? If this were encountered the relative degree of cross-protection which naturally should always be carefully controlled might reveal differences. Theoretically at least, there is no reason to believe that apparently complete mutual cross-protection might not occur with two closely related but not identical strains. If this is theoretically possible it again brings us to our previous theorem that *antibody absorption is our ultimate criterion.* This has been discussed primarily under agglutination, because agglutinin absorption is usually the expedient method since we can control the technic more easily and because quantitative estimations can be more easily and accurately made. The same principles, however, are applicable to the absorption of other antibodies. The possibility of normal antibody giving what appears to be "group" protection must not be lost sight of.[1]

Other Methods of Determining Protection.—Animals may be injected with a vaccine, and the protective power conferred measured by the resistance of the animal to a fatal dose (or multiples) of a virulent organism of the same type. This method may also be employed to study the degree of cross-protection, conferred by related types. Here again cross-protection is not necessarily a criterion of identity.

Active Immunization—Vaccination.—The first vaccine employed was cowpox vaccine, a living virus, which caused vaccinia at the site of inoculation with resulting immunity to smallpox. The term vaccine has its derivation from the Latin, *vaccinus,* relating to a cow, but usage has broadened the term to include any active immunizing preparation of a virus or microbe without regard to its origin. Thus, the rabies vaccine, the virus being non-cultivable, is made from the brain of a

[1] For antistreptococcus serum testing, see Hyg. Lab. Bull., 1923, **134**, 35.

rabbit dying of rabies, that is, it consists of the virus and rabbit tissue. Some employ the term vaccine in a still broader sense, and apply the term to diphtheria—toxin-antitoxin or toxoid—and to the scarlatinal toxin used to produce antitoxic immunity. The term bacterin is used by some to designate bacterial vaccines. The first successful bacterial vaccinations were those of Pasteur, who immunized fowls against fowl cholera and sheep against anthrax by the use of attenuated cultures of the respective bacilli.

Bacterial Vaccines.—The rational application of bacterial vaccines as specific agents (antigens) presupposes a knowledge of the bacterial etiology of the infection or disease and the immunological character of the etiological agent. Thus, is the infection due to a single type, to several or more types or to a multiplicity of varieties. A knowledge of the immunological types or varieties which are included under a single bacterial name is essential.

In preparing bacterial vaccines it is also essential that dominant type strains be selected. Stock strains should be tested before use, to be sure that they are typical, that no evidences of degradation are present. (See under Agglutination.)

Types of Bacterial Vaccines.—*Killed Bacteria.*—This is the one most commonly employed. Heat is usually the means. Overheating is to be avoided, as it may lower the antigenic value. The minimum time and temperature necessary to kill should be employed. In the case of the enteric vaccines less than this is used, the residual living organisms being killed by the subsequently added preservative. Carbolic acid or tricresol have been advised as killing agents. There is no definite evidence that this procedure is superior. Recently the presumption has been raised that heating might destroy an essential toxin contained in some vaccines. This is purely hypothetical.

Bacterial Extracts, Autolysates, Digested Bacteria.—The object of such preparations is to hasten absorption and to bring into solution the toxic substance of the bacterium. Such preparations are likely to give severe or even serious reactions and should be used with caution. The question also arises as to how far such preparations retain their specificity (see Specific Constituents under Agglutination). The favorable curative results reported may be due to non-specific protein action.

Live Bacteria.—Attentuaed bacteria, as in the Pasteur anthrax vaccination method, are used. The use of such vaccines in man has been limited. The Calmette attenuated tubercle bacillus vaccine is the only one now in use. (See Tuberculosis.)

Sensitized Vaccines.—Bacteria treated with immune serum to lessen or avoid local and general reactions have been recommended by Besredka and others. Their superiority over ordinary vaccines is not generally accepted. Simultaneous injection of serum and vaccine has also been recommended.

Preparation of Bacterial Vaccines.—The following outlines the usual procedure: Cultures are grown on agar or in broth. The growth from agar is washed off with saline or, if serum or blood be required in the

medium, scraped off into saline. Broth cultures should be sedimented in the centrifuge and the sediment resuspended in saline. The suspension is shaken thoroughly to obtain even distribution and a sample removed for standardization. The container is then closed with a sterile rubber stopper which should be tied in, and the whole container submerged in a water-bath and heated to kill the bacteria. Not over 55° C. should be employed; not over 53° C. for the typhoid vaccine. Heating is usually for one hour. A preservative, carbolic acid or tricresol, 0.3 per cent, is then added. Subsequent dilution to the appropriate strength is done with saline containing the same amount of preservative.

Standardization of Bacterial Vaccines.—Vaccines may be standardized by weight, by count, by turbidity or by volume. The most commonly used procedure is by count. A direct count may be made by means of the blood platelet counting chamber. An indirect counting method is that of Wright. Turbidity may be measured by the various types of nephilometers. The volume of bacteria is best measured in the special centrifuge tube of Hopkins.

Should there be any objections to handling the sample withdrawn for standardization because of the presence of living bacteria, 1 per cent. of formalin may be added. This will not interfere with the staining capacity and limits autolysis.

The Wright method is sufficiently accurate for practical purposes and is done as follows:

A capillary pipette (see p. 276) is marked about one inch from the tip. The finger is pricked and blood drawn up to this mark; a bubble of air is then allowed to enter the tube and the bacterial suspension drawn up to the mark. The contents are then mixed on a slide and thin films (as for blood) made and stained. On the under lens of the eye-piece a one-quarter-inch square is marked out with a pencil, and using an oil-immersion objective the number of bacteria and red cells appearing in this square are counted separately. About fifty squares are counted and the average per square obtained. The number of red cells per cubic centimeter being known, the number of bacteria are obtained according to the following proportion: Number of red cells per square : number of bacteria per square : : 5000 (millions) : × (millions).

Testing the Potency of Bacterial Vaccines.—Two methods are available: (1) The determination of the degree of protection developed by vaccinated animals; (2) the determination of the antigenic value in terms of the antibody response following injection of man or animals. While it is not established that such tests are necessarily a criterion of the actual immunizing value in man, the insistence upon such tests by the controlling authorities (Hygienic Laboratory) is of great importance. Such tests will increase the care in production and will prevent sophistication, intentional or unintentional, through the use of cultures not true to name. It is further a preventative against the use of degraded cultures. An antigenic standard has been placed on typhoid vaccine, the agglutinin response being used for expediency. Similar standards should be placed upon other vaccines. In some instances it will probably be necessary to employ the protection method (pneumococcus).

Length of Potency of Vaccines.—This has been studied by determining the antigenic response in animals.

Typhoid vaccine kept at ice-box temperature shows little apparent deterioration until after eighteen months storage.[1] Recent investigations in this laboratory show that there is no apparent deterioration of pertussis and pneumococcus vaccines even at the end of two years.

Application of Bacterial Vaccines.—Bacterial vaccines are used as prophylactic and as curative agents. Their use for prophylaxis is based on the specificity of the vaccine in relation to the etiology of the disease to be prevented. In the curative use two factors must be considered—specific antigenic capacity and the non-specific effect of foreign protein. The use of specific bacterial vaccines in the course of an infection was primarily based on the conception that the infection may not be sufficient stimulus to the production of adequate curative antibodies. The additional stimulus of vaccines it is believed will increase the antibody production and hasten cure. On this basis, localized lesions of a subacute or chronic character should be the type of case most benefited. Although this is, in general, the fact, the more acute and general types of infection are also benefited in some instances by vaccine therapy. It would seem that when the blood stream was invaded by bacteria all the tissues of the body would be stimulated to their maximum capacity of response. We must, however, distinguish three types of generalized invasion: (1) Where there is an initial blood invasion with secondary localization and the disappearance of the infective agent from the blood; (2) acute local infections, with limited invasion, without multiplication in the blood (simple bacteriemia); (3) a septicemia, invasion and multiplication in the blood stream.

The first two show the ability of the body to limit the blood infection by a response of the protective forces of the body. This, however, may not be the maximum possible response and in these conditions vaccines do increase the antibody content of the blood, an example being typhoid fever. Furthermore, the injection of vaccines may lead to a cellular response in the form of a polymorphonuclear leukocytosis, or an increase if already existent. It is evident, however, that injudicious dosage might overwhelm the body and break down the ability to respond, with serious consequences to the patient. In the septicemia types, intravascular multiplication with no tendency to localization usually indicates the inability of the body adequately to respond, and little is to be expected from the further stimulus of vaccines. This is usually found to be the case.

Considering specific response to vaccines, one would not expect much benefit in acute self-limiting types of infection, as the antibody response requires several days to become marked.

Theoretically, if the antibodies cannot reach the focus of infection in sufficient concentration, vaccines would be of little help. This is probably a factor in the generally poor results obtained with infections of bone sinuses or cavities.

[1] McCoy and Bengston: Hyg. Lab. Bull., 1920, **122**, 7.

Vaccines are an aid, not a substitute, for indicated surgical procedures, and the application of the latter should never be delayed. Incision, drainage and relief of tension, even if pus has not formed, are of curative value due partly to better circulation, also to the fact that drainage allows the exudation of fresh serum and cells, both of the utmost importance.

Non-specific Response to Specific and Non-specific Vaccines.[1]—The occurrence of prompt curative response following the injection not only of specific but also of non-specific substances cannot be explained on the basis of specific antibody response. Such a response, especially following intravenous injection of non-specific substances would at first glance overturn all our ideas of the specific action of therapeutic vaccines. A method of treating typhoid fever by intravenous inoculation, though little used today, is a good example. The injection of an appropriate amount of typhoid vaccine or even colon vaccine or albumose, is followed by a chill and a rapid rise of temperature followed by a progressive fall, sweating and marked subjective and general improvement. At first there is a decrease, then a sharp increase of the polynuclear leukocytes. In some cases the reaction develops into a recovery by crisis, in others the disease resumes its course and a second injection, giving a similar reaction, may or may not be followed by critical recovery. Similar curative reactions have been obtained in other infections.

As far as we know at present, the following factors probably enter into such curative reactions. The sharp leukocytic response is undoubtedly of value. Increased antibody production cannot quickly develop unless it has started, thus vaccination during the incubation period of typhoid fever has little, if any, effect on the subsequent disease, which should be the case if the mere increase of antibodies were the only important factor. The best results are obtained in typhoid fever after the tenth day of the disease, that is, after response to infection has been fully established and the injection at this time is followed by an increase of the antibodies in the blood. What apparently takes place is a stimulation of the hemopoietic organs (the probable source of antibodies) with the release and dispersion of antibodies already formed and the throwing out of polynuclear leukocytes. A further factor as pointed out by Jobling is the mobilization of non-specific ferments, the serum-protease, which may reduce the bacterial products to non-toxic state. A reaction seems essential if definite curative effect is to be obtained.

The further details concerning vaccines are given in the chapters on the individual species or types of bacteria.

[1] For a full discussion and bibliography, see Peterson: Protein Therapy and Non-specific Resistance, Macmillan, 1922.

CHAPTER XIV.

HYPERSENSITIVENESS.[1] THE BACTERIOPHAGE OF TWORT (D'HERELLE).

In preceding chapters we have considered the production of immunity through the introduction of antigenic substances. We have now to consider a phenomenon which seems at first to be the antithesis of immunity. Thus, if a very small amount of microbal or other protein be injected into a guinea-pig, a change occurs so that while the first dose produces no noticeable reaction a second injection of the same protein given ten to fourteen days later will cause symptoms of shock or even death of the animal. This phenomenon Richet called "anaphylaxis." This reaction in the guinea-pig has been studied in detail with the hope that the evidence acquired would help explain somewhat similar phenomena encountered in infectious diseases as well as the reactions following the repeated injections of antitoxins or serums, ingestion of certain food or drugs (idiosyncrasies), etc. Furthermore, this phenomenon has been compared with the development of the common symptoms (fever, etc.) of infections.

There are differing views as to the classification of, and the terminology applicable to the phenomena to be described. The following is that of Coca.[2]

Specific Hypersensitiveness.

Anaphylaxis.	*Allergy.*
	Hypersensitiveness of Infection.
	Specific Dermatitis.
	Atopy.
	Serum Disease.
	Drug Idiosyncrasy.

This classification and the arguments advanced in its favor serve to emphasize the differences between the phenomena under discussion. To some extent the attempts to develop hypotheses, and to apply these hypotheses too generally, has tended to obscure these differences. The separation of the phenomena with appropriate terminology has a direct practical bearing. Thus the use of the term anaphylaxis to designate all the reactions seen in man following the introduction of antitoxin or serum has led to an undue fear of such reactions, as in the minds of most the term implies acute shock and possible death.

[1] For fuller discussion and bibliography see:
Coca, A. F.: Hypersensitiveness, Tice's Practice of Medicine.
Vaughan, V. C., and Vaughan, W. J.: Protein Split Products in Relation to Immunity and Disease.
Duke, W. W.: Asthma, Hay Fever, Urticaria and Allied Manifestations of Allergy.
Also references cited previously, Chapter VII.

[2] Jour. Immunol., 1923, **8**, 163, and personal communications.

Anaphylaxis in Guinea-pigs.—Theobald Smith found, when he attempted to give an injection of horse serum to guinea-pigs which had been used for antitoxin testing some weeks before, that striking symptoms developed and that fully half of the animals died in thirty minutes. Untreated animals showed no reaction from the same dose. Rosenau and Anderson and Otto studied this phenomenon and showed conclusively that the antitoxic content of the serum was not a factor, but that the symptoms were wholly due to the serum proteins. It was found that an incubation period was necessary before the pig became hypersensitive and that this period became longer the larger the initial or sensitizing dose. Very small amounts of serum may suffice to sensitize. Doses of 0.001 to 0.01 c.c. will more uniformly sensitize. The size of dose required to produce an anaphylactic reaction depends on the rapidity of absorption. Thus a small dose will kill when given intravenously; a larger dose is needed if injected intraperitoneally and still more if given subcutaneously. The rapidity of development and the severity of the symptoms after the second injection depend on the sensitiveness of the animal and the size and mode of administration of the dose. Intravenous (intracardial) doses of 0.01 c.c. will elicit symptoms; larger doses, 0.1 to 0.5 c.c., will usually kill. The guinea-pig first shows restlessness, then scratches at the mouth, coughs and develops spasmodic or rapid breathing. Urine and feces are discharged. The guinea-pig then falls on its side, breathing becomes difficult or arrested and spasmodic or convulsive movements supervene. The convulsions are usually followed by the death of the animal. Associated with these symptoms there are a fall in temperature, a leukopenia, a diminution of complement in the blood and a delay or loss of blood coagulability. On autopsy the lungs are markedly distended, due to stimulation and contraction of the smooth muscle of the small alveoli, thus retaining the inspired air. This explains the respiratory symptoms noted before death. The heart continues to beat for some time after death. The contraction of smooth muscle is due to peripheral or cell irritation.

In Rabbits.—It has been estimated that rabbits are $\frac{1}{400}$th as sensitive as guinea-pigs. Symptoms of anaphylaxis may be produced easily, but acute anaphylactic death occurs only irregularly. Several injections of serum are needed to sensitize the animal. Sensitization is most constantly produced when several subcutaneous doses of 1 or 2 c.c. are injection at five-day intervals and followed three days after last injection by daily intraperitoneal or intravenous injections of 0.2 c.c. for two weeks or more. The shock injection of 2 c.c. is given five days later by the intravenous route. The symptoms elicited are less marked than those observed with the guinea-pig and the intense dyspnea is absent. Death is due to cardiac and vascular disturbance. The pulmonary arterial system is so contracted that perfusion of salt solution even under high pressure is impossible.

Besides acute anaphylaxis, Arthus has demonstrated a peculiar local effect following repeated doses of serum. If several or more injections of serum are given a rabbit, subsequent subcutaneous injections will

frequently cause severe local reactions which may result in local necrosis (Arthus's phenomenon). This is not a cumulative effect, as previous subcutaneous injections caused no reaction even when given in the same place, whereas the later reaction occurs wherever there is an area of injection.

In Dogs.—Dogs may be sensitized with two injections of serum (first subcutaneous, second intravenous) five days apart. A shock dose of 20 to 30 c.c. given intravenously several weeks later will cause symptoms and death. Vomiting or retching and evacuation of the bowels are among the earliest symptoms. The circulatory disturbance is apparently due to engorgement of the liver resulting in a great accumulation of blood in this organ and in the portal vessels. This would account for the anemia and low pressure in the general circulation.

Dependence of Symptoms on Contraction of Smooth Muscle.—Schultz has demonstrated that the hypersensitiveness of smooth muscle is the main factor in anaphylactic response. In the guinea-pig this is shown by the contraction of the bronchial musculature. In rabbits and dogs this bronchial phenomenon is not marked, because the bronchi are relatively poorly supplied with muscle. The circulatory disturbances in the dog and the rabbit are extreme contractions of the blood vessels. The cause of the disturbance is the same, though there is a difference in the site of action. This effect on smooth muscle can be demonstrated on excised muscular organs, such as the uterus.

Sensitization by Other Routes.—In the preceding paragraphs only sensitization by injection has been mentioned. Sensitization induced by feeding has been reported by Rosenau and Anderson. Other observers have had contradictory results. Ratner has confirmed it. It has been shown that guinea-pigs may be sensitized by inhalation of antigenic sprays and may be shocked by the same route. Ratner[1] has demonstrated the same thing with powdered antigens.

Passive Anaphylaxis.—In contrast to the active sensitiveness, hypersensitiveness of one animal may be transferred to another by the injection of the blood or serum of the first animal. This is not only possible with animals of the same species (homologous, viz., guinea-pig to guinea-pig), but with animals of different species (heterologous, viz., rabbit to guinea-pig). Hypersensitiveness is transferred to the offspring of sensitized mothers; thus, young guinea-pigs may be sensitive for five or six weeks or longer after birth. Active sensitization may also occur through placental passage of the antigen.

Anaphylactic symptoms cannot be induced in a guinea-pig by the injection of antigen, until some time (four to six hours) after the immune serum has been given. In the rabbit and dog it may be induced immediately after injection of the immune serum.

The Anaphylactogen.—This term is applied to the substance which induces the state of anaphylaxis. All the available evidence indicates that only proteins which can act as antigens may be anaphylactogens. The proteins may be of animal or plant origin, including microörganisms

[1] Jour. Immunol., 1927, **14**, 303; Proc. Exp. Biol., 1928, **26**, 3.

and their extracts. Such proteins must be alien to the animal injected or at least alien to his circulation.

Probably the whole protein molecule is necessary to induce hypersensitiveness. Proteins split by digestion or chemical agents lose the sensitizing property. Heating diminishes or destroys the sensitizing action of proteins in proportion to the degree of coagulation caused by the heating. The ability to produce anaphylactic shock may be lost before the ability to sensitize, because minute residues of the original protein would still suffice for sensitization. Vaughan believes that the protein possesses a non-specific toxic portion and a specific sensitizing portion. He obtained two such fractions by treatment with an alcoholic solution of alkali. The sensitization by the one fraction could equally be used as an argument that all of the protein molecules had not been cleaved. The anaphylactogen, at least with native proteins and bacteria, is specific, that is, only the same protein which was used for sensitization will cause anaphylactic symptoms. This specificity follows, as a rule, the biological origin, as has been noted with other antigens.

Similar Non-specific Phenomena.—The products of protein cleavage when injected intravenously in normal animals are toxic. An example has been noted, viz., that obtained by Vaughan. Peptone likewise causes the symptoms of anaphylaxis. Bordet showed that the addition of agar jelly to guinea-pig serum and subsequent incubation at 37° C. rendered the serum toxic. In fact, many substances, chloroform, colloidal silica, kaolin and even distilled water, will render normal or immune serums toxic. This toxicity manifests itself by causing anaphylactic symptoms. The same effect may be noted after intravenous injection of such substances, the toxification of the blood occurring *in vivo*. Novy has found that blood in the precoagulation stage is likewise toxic. These results have a bearing on the explanatory theories of anaphylaxis. Our acceptance of a theory will depend on whether or not we agree that the similar response is proof that the poison in these non-specific phenomena is the same as in specific anaphylaxis. Manwaring believes that the physiological basis of peptone shock and anaphylaxis in the dog are different.[1]

Anti-anaphylaxis or Desensitization.—If animals survive the second dose of protein they are for some days relatively very insensitive to the protein. The injection into the guinea-pig of one or more small doses of the specific protein during the period of incubation has the same effect. This condition of desensitization is only temporary, sensitization again developing after several weeks. The serum of an anti-anaphylactic animal does not confer this property to a second animal. In fact, such a serum may passively sensitize if the desensitization is not complete. The injection of an immune serum (specific for the protein) prior to the dose of protein will interfere with development of anaphylactic symptoms. A relative resistance to anaphylaxis may also be induced by the injection of non-specific substances, proteins, peptones, etc., as well as with drugs, atropin, chloral, etc.

[1] Jour. Immunol., 1923, **8**, 191.

Theories of Anaphylaxis.—Two theories have been advanced which assume that the toxic agent is a digestion product. Vaughan believes that the first injection sensitizes the animal by stimulating the production of specific zymogen which when activated will cleave the protein on second injection, the products of this cleavage being toxic. The zymogen according to this conception would be an antibody which would be activated by the complement. Friedberger maintains the same views, based on his observations that substances inducing anaphylactic-like shock develop in test-tube mixtures of antigen and its antiserum; the term anaphylotoxin is used to designate the shock-inducing poison.

This conception involves the idea that amboceptor and complement activity results in proteolytic cleavage. Jobling found no evidence that immune body and complement will cause proteolytic cleavage. Nor is there reason to believe that there is produced some other specific antisubstance having actual proteoclastic activity. To sustain his theory, Vaughan concludes that the anaphylotoxin and the poisons resulting from proteolytic cleavage are the same, because they cause the same physiological effect. By the same reasoning he would be forced to assume that the toxic products in a mixture of serum and a non-specific non-protein substance (see above) where no protein antigen is available for cleavage are again the same poison. Friedberger's idea that complement is essential to the reaction is also untenable for similar reasons.

Jobling in his earlier work met these objections by the hypothesis—that the digestion products come from the serum and not from the antigen, this digestion resulting from the depression or neutralization of the antitryptic substances which would allow the serum-protease to digest the serum. Non-specific substances as mentioned above would cause this effect because of their neutralizing action. Specificity could be assumed on this basis to be due to the fact that the antigen-antibody complex would possess this neutralizing action.

Both digestion theories fail in our opinion to explain the quick response especially of excised smooth muscle. Dale employing the reaction of the uterine muscle, has questioned whether toxic digestion products could be produced within the time between contact of antigen and muscle response. The rate of reaction is equal to that of a preformed protein poison such as histamin. Digestion theories fail to interpret the delay in passive sensitization of guinea-pigs.

Novy and De Kruif have elaborated a theory, drawing an analogy between the production of anaphylotoxin and the mechanism of blood coagulation. This latter results from the change of fibrinogen into fibrin through the so-called fibrin ferment. They assume the existence of a more or less labile matrix in the blood which is changed by a catalyzer into anaphylotoxin. The two phenomena are similar, therefore, in that a labile substance undergoes tautomeric modifications, on the one hand the insoluble fibrin, on the other, the soluble anaphylotoxin. This theory assumes that the non-specific substances noted above give

rise to the same poison as is active in specific anaphylaxis. The only difference is that the antigen-antibody complex is the catalytic agent in specific anaphylaxis. This theory also fails to explain all the phenomena, especially the delay in passive sensitization. It does seem to eliminate the doubtful aspects of the digestion theories.

A moderate toxicity of the blood has been demonstrated by Novy for animals sensitized and shocked by relatively large doses of antigen or by non-specific agents. The question arises would this be so if the minimal shock-inducing dose had been used. Weil who transfused normal dogs with the blood of dogs dying of anaphylactic shock uniformly failed to demonstrate any increased toxicity. If the toxic products were formed in the circulating blood, one would expect to be able to demonstrate at least some toxicity.

If the digestion theories or theory of Novy assumed the cell as the site of toxin production, the time factor raised by Dale would still be a difficulty.

All the theories thus far given do not take into consideration the fact that the organs of an immune animal are hypersensitive although the animal as a whole is not. This as well as the fact that the injection of immune serum specific for the protein to be used will prevent anaphylactic shock, indicates strongly that an excess of antibodies in the circulating blood acts as a protective barrier, preventing the antigen from reaching the cells.

Because of the facts given above the opinion is being accepted that the site of the production of the toxic substance is in the cells. This is spoken of as the cellular theory. Many observers believe that the antigen combines with the cellular antibodies and that the combination in some way injures the susceptible cells. Such a theory would be compatible with the delay in passive sensitization in guinea-pigs, the latent period being assumed necessary for combination of the introduced antibody with the cell.

The question as to the nature of the antibody involved brings up the question of the unity of antiprotein antibody.[1] The production of anaphylaxis in guinea-pigs in which complement is suppressed has been advanced as proof that complement is not involved in the reaction. The absence of demonstrable complement in the blood is not necessarily a demonstration of its absence in the cells. Most of the adherents of the cellular theory believe that the precipitins are involved in the reaction. The evidence advanced in favor of this idea is that the capacity of serum to confer passive hypersensitiveness is proportionate to its precipitin content. If a cellular theory as outlined is accepted, the specific anaphylotoxin must be assumed to be distinct from the toxin produced in the test-tube by admixtures of specific antigen and antiserum or of serum and non-specific substances, as well as from the products of protein digestion.

On the whole one cannot but conclude that no hypothesis is satis-

[1] See Chapter VII.

factory which does not place the major emphasize on the cell as the probable seat of the reaction.

Anaphylaxis and the Symptoms of Infection.—Vaughan's theory of anaphylaxis has received such wide attention and acceptance because it seemed to explain in a satisfactory manner the toxic symptoms of infection caused by bacteria producing no demonstrable extracellular poisons. According to this conception the digestion products of the microbal protein would be the active agent. Carrying this conception further, the poison would be non-specific in character, being developed in the cleavage of any protein. Vaughan has shown that fever and the accompanying symptoms noted in all infections can be reproduced by the injection of protein cleavage products, the character of the fever depending on the size and number of doses injected. The hypothesis of Friedberger is very similar. The modified theories of anaphylotoxin production of Jobling or that of Novy could be similarly adapted as an explanation. Although there is strong doubt that digestive products play any rôle in the development of the anaphylactic reaction, the objections raised would not hold true in relation to the symptoms of disease. In the absence of more opposing evidence it seems probable that digestion products, although not due directly to antibody action may play a partial role at least in the production of disease symptoms. If, as seems implicated in such hypotheses, antibodies are necessary for the development of the poison it is difficult to understand the symptoms associated with very acute or fulminating infections.

Alleged Toxin Hypersensitiveness.—Because of certain phenomena, some investigators believe that there develops at times a hypersensitiveness to soluble or exotoxins, comparable to some extent with the hypersensitiveness to bacterial protein. As has been noted in the previous chapter, if an M. L. D. of toxin is divided into ten or twenty parts and one part injected every day or every other day, death will take place when about 40 to 60 per cent. of the M. L. D. is given. The animals do not die quickly after the last dose but after one or more days of increasing weakness. It has also been observed that if several small doses of toxin are given, fifteen to twenty days later before any trace of antitoxin is manifest, additional doses of as little as $\frac{1}{100}$th M. L. D. will cause a slow death. Suggested explanations are that either certain cells stimulated by the previous injections have developed a great avidity for the toxin or that some cells naturally possess a greater avidity. In either case, there being little toxin, they absorb the major part of the toxin or in the aggregate as much as when a larger dose is given at one time.

Behring has made the statement that horses under immunization possessing large amounts of antitoxin in their blood showed hypersensitiveness to the toxin. These animals, however, did not develop symptoms referable to the specific action of the toxin injected. Doerr, Pick and others have pointed out that as we never inject toxin in a pure state, the symptoms were most probably due to the associated meat extractives, peptone and products of bacterial autolysis present in the toxic broth.

We believe that this is the true explanation. It seems to be sustained by the appearance of same type of reaction when horses are immunized by the injection of broth cultures of non-toxin-producing bacteria. We have immunized over 700 horses to diphtheria toxin and over 50 horses to tetanus toxin and have never encountered a reaction which appeared to be toxin hypersensitiveness after antitoxin had developed. This is true also for guinea-pigs.

We have already referred to the long delay which intervenes between the injection of toxin and the development of antitoxin in the guinea-pig or rabbit. This seems to have misled many observers, thus Loewis and Meyer reported that small animals which have no natural antitoxin will show no response to a single injection of a toxin-antitoxin mixture, but will respond with antitoxin development if a second dose is given some weeks later. Our own observations show that the great majority of susceptible animals will in time develop at least some antitoxin after a single dose. As has been pointed out, in a previous chapter, if antitoxin has developed because of some known or unknown cause, a subsequent injection of toxin or of a toxin-antitoxin mixture will cause a quicker and greater response than in cases where no antitoxin is present. Hypersensitiveness of a different kind exists, therefore, in the sense that the ability of antitoxin production is increased.

Hypersensitiveness of Infection.—In many infections there develops in variable degree a hypersensitiveness to the substance or products of the infecting type of bacterium. The most marked examples of this are noted in infections due to the tubercle bacillus and to the glanders bacillus. Tuberculin which consists of the soluble products found in a broth culture of the tubercle bacillus will serve as an example for discussion.

Tuberculin Hypersensitiveness.—Tuberculin is appreciably toxic only for an animal which, through natural infection or by injection of live or dead bacilli, has developed tubercle tissue. This hypersensitiveness is shown by the skin, the mucous membrane or by a systemic as well as by a focal reaction (site of lesion) when tuberculin is injected in sufficient doses. If the dose injected be sufficiently increased death of the sensitive animal is caused. The substance or substances involved are highly resistant to heat, and are specific but not anaphylactogenic, that is, animals cannot be rendered sensitive by tuberculin injections. A relative tolerance can be induced in the tuberculous animal by injecting gradually increased doses. The proteins of the tubercle bacillus are anaphylactogenic, but the hypersensitiveness to these, which can be induced in normal animals, is a different phenomenon. The mechanism of the tuberculin reaction is obscure. (See Tuberculosis.)

Hypersensitiveness in Other Infections.—A skin reaction is elicited in a considerable proportion of cases of syphilis by the intracutaneous injection of "luetin," an emulsion of the Treponema pallidum. Similar indications of hypersensitiveness, ophthalmic or cutaneous, are noted in typhoid fever or following the injection of typhoid vaccine. Positive skin reactions have also been elicited by the gonococcus and by some

of the fungi. These reactions are relatively specific but not sufficiently so to be diagnostic. The practical value of the skin reactions other than that of tuberculin and mallein is materially reduced because of the relatively frequent occurrence of non-specific skin hypersensitiveness, which leads to confusing reactions. With our present knowledge we are not justified in assuming an identity in the nature of these reactions and that of tuberculin.

Hypersensitiveness and Immunity.—Jenner observed the more rapid appearance of a reaction following vaccination after a previous vaccinia. This observation has been studied by others and the evidence indicates that such an accelerated response is an indication of immunity. In the case of tuberculin, the reaction is also indicative of immunity to reinfection. The disappearance of the reaction in a person with a latent lesion, during measles for instance, is not infrequently followed by extension of the lesion and tuberculous disease.

Because of the immunity-index character of the tuberculin reaction and the accelerated reactions observed in vaccinia, the conception that active immunity is basically a sensitization of the body cells has been advanced. This would operate when after a lapse of time all demonstrable antibodies had disappeared from the blood. This sensitization of the cells it is believed is due to an acquired temporarily and quantitatively exaggerated ability of response, the acquisition of this capacity being due to the stimulus of the immunizing agent. The basis of this ability we may conceive as due to the persistence of cellular antibodies. Stimulation may result also in the release of the antibodies as well as a rapid reproduction. In the case of antitoxic immunity this is actually demonstrable. In the case of the antimicrobal types of immunity the results of injections of vaccines in immune individuals indicates that the antibody response is greater in the immune than in the normal individual. The results of some observers have shown striking differences. Non-specific factors are involved to some extent in this difference. The injection of a non-specific vaccine, according to Jobling, will cause a dispersion or release of preformed antibodies, thus increasing the blood content. Gay attempted to show that immune typhoid rabbits responded with a specific leukocytosis, that is, a specific cell-production stimulation resulted when injected intravenously with typhoid vaccine. McWilliams was unable to verify these results.

Atopy—Hypersensitiveness to Foods, Pollens, Etc.—This phase of the subject includes the hypersensitiveness to pollens and other substances, which hypersensitiveness is manifested by asthma, hay fever and specific eczema. Some of the peculiar idiosyncrasies to foods insofar as manifested by gastro-intestinal symptoms seemingly fall in this category. The inducing substances "atopen" (Coca and Cooke[1]) may or may not possess antigenic value. All the evidence available indicates that atopy is an inherited predisposition. Thus Cooke[2] has found the condition

[1] Jour. Immunol., 1923, **8**, 163, and personal communications.
[2] See also confirmation by Balyeat: South. Med. Jour., 1928, **21**, 554.

present in 71 per cent. of the offspring where both father and mother give a history of the condition. According to the Mendelian law, if the condition were a dominant gen it would appear in 75 per cent. Identical twins develop the condition at the same time and to the same substance. Although the condition is inherited, the sensitiveness to a definite substance is not necessarily the same in parents and offspring. Of course, exposure is the determining factor in the development of the specific reactivity and this may differ in different members of the family. Prausnitz and Küstner, de Besche and more recently Coca[1] have shown that the intracutaneous injection of serum from most sensitive individuals into a person not sensitive will cause a local hypersensitiveness to the substance to which the former is sensitive. On this basis Coca postulates the existence of a specific substance in the blood, the "reagin."

Specific antiserums, to egg-white for instance, will not sensitize the skin. Conversely the "reagin" containing serum will not passively sensitize the uterus of a guinea-pig. The reagin containing serum will not "neutralize" its homologous atopen, nor has it any precipitating action. It is only produced in the sensitive body, yet it has some similarity to the antibodies described in preceding chapters in that the amount is increased during treatment viz: injection of the atopen. There is one strange exception: persons who are worm-infected develop reagin substances, yet present no symptoms of hypersensitiveness. The worm antigen stimulates the production of specific reagin in all persons and at any age. Reagins seem to be consistently absent from the blood in idiosyncrasies expressed by urticaria, angioneurotic edema, provided symptoms of mucous membrane involvement are absent.[2] Coca believes that this group may be analogous to serum sickness, the absorption route being the intestinal tract.

Atopy is accompanied by a specific skin sensitiveness to the atopen. The mucous membrane of the eye is even more exquisitely sensitive. Injection (or ingestion) of the atopen may lower the clinical sensitiveness and this is employed practically in the prophylactic treatment of hay fever and asthma or to reduce the sensitiveness of certain foods. Recent observations indicating that the injection of horse serum will apparently induce a skin sensitiveness to horse serum should be mentioned. This phenomenon does not belong in the category of atopy. The facts given concerning atopy show that this phenomenon has only some similarity to anaphylaxis in experimental animals.

Specific Dermatitis.—Susceptibility to poison ivy, poison oak, primrose, etc., is more or less developed in a majority of humans. Animals are immune. The active principle of poison ivy is an oleoresin which has been used as a therapeutic or preventive measure; that is, injection or ingestion will materially lower the sensitiveness to these plant poisons.[3]

[1] Proc. Soc. Exp. Biol. and Med., 1923, **21**, 49.

[2] For outline of food allergy and references see Rowe: Jour. Am. Med. Assn., 1928, **91**, 1623.

[3] Jour. Am. Med. Assn., 1923, **80**, 1588.

Serum Sickness and Similar Conditions.—The injection of antitoxin globulins, antiserums or other substances may cause untoward reactions. These reactions have nothing to do with the antibody content. Following the first injection, three types of reactions may be observed: (1) collapse, with or without fatal outcome; (2) the symptom-complex called "serum sickness;" (3) local necrosis. The same form of reaction may follow a second or later injection.

Collapse or Death.—In about 1 in 20,000 primary injections more or less alarming symptoms develop; in about 1 in 70,000 injections death results. The symptoms develop quickly and are those of extreme dyspnea and collapse. Small amounts of serum in these hypersensitive people may suffice to cause death, probably as little as a few drops of serum given intravenously may be fatal to some individuals. Kerley found in the case he reported that as little as 4 minims given subcutaneously gave alarming symptoms. Atopic persons, those with hay fever or asthma, sensitive to horse products, may be expected to react badly. Some children with status lymphaticus have died, and it has been generally believed that they are bad risks. Whether these children were also hypersensitive is not known.[1] Fatalities occur most often after primary injections. There are of course more of these.

A relatively more common form of reaction is noted in about 40 per cent. or more of the cases injected intravenously with an antitoxin or antiserum. This consists of a chill, more or less severe even though the product injected be warm and is injected slowly. This occurs with some preparations and not with others, with the serum of one horse and not with that from another. The reason for its occurrence is obscure.

In some instances, the intravenous or intraspinous injection of antitoxin globulin or antiserum several weeks or longer after an initial injection which caused no marked reaction, has resulted in alarming symptoms of collapse or in a few instances in death. This effect almost never follows a second subcutaneous injection. There may be, however, an immediate development of serum sickness. If after several injections a period of several days is allowed to elapse, an intravenous injection is likely to give a marked reaction. Why this peculiar intolerance should develop is not clear.

Serum Sickness.—There is no evidence that previous exposure is an element in this phenomenon. With the ordinary limits of dosage, the incidence ranges from 10 to 60 per cent., the higher incidence following the larger doses. Because of the lower total solid content, and also because of the heating, refined concentrated antitoxin is less likely to cause reactions.

Following the first injection of serum or antitoxin there is an incubation period varying from three hours to twenty-four days. Most commonly the period ranges from seven to twelve days. The symptoms are primarily a skin eruption, edema, slight albuminuria, variable both in

[1] For summary of fatal cases see Lamson: Jour. Am. Med. Assn., 1924, **82**, 1091.

incidence and in degree, enlargement of the lymph nodes with pain and tenderness, and pain in the joints. Following the prophylactic use of scarlatinal antitoxin, neuritis has been observed in several cases. The eruption is very variable in character. A local eruption appears earlier than the general eruption. On the second or later injections the period of incubation may be absent ("immediate reaction") or shortened ("accelerated reaction"), although this does not always occur. This condition is not serious, and in many instances gives no greater discomfort than any itching rash. Some samples of serum or antitoxin uniformly cause a skin eruption earlier than others and of a peculiar type, such as scarlatiniform. The longer incubation periods are more frequently followed by urticarial rashes. It would seem from this that there were different reaction-inducing substances in serum.

Von Pirquet and Schick, who have studied this condition in detail, believe that the reaction is due to antigen-antibody combination. They point out that the average incubation period coincides with the time of the first appearance of precipitins in experimental animals. Likewise, they believe that the immediate or accelerated reactions following later injections are explained by the presence of developed or developing antibodies. This explanation apparently does not account for the fairly common occurrence of a short incubation period.

Local Reactions.—In very rare instances, the primary injection of antitoxin leads to local necrosis. Although this occurs with extreme infrequency it should be a warning against injection under the breasts. When repeated injections are given, a final subcutaneous injection somewhat more frequently results in a sharp local reaction which may go on to necrosis. This may occur not only with serum, but also with rabies vaccine. The necrosis is not due to bacterial contamination but the necrotic area may become infected and serious or fatal results ensue.

Desensitization to Serum.—Instances are noted with some frequency where first injections have caused reactions and following injections of the same size given up to several days later produced no reactions. This would seem to indicate the development of a tolerance or, to use the term usually applied, a desensitization. As was noted, different batches of antitoxins or antiserums may differ widely in the reaction-producing qualities, and this tolerance may be wholly apparent. A tolerance seems to be established in some instances by starting with the injection of a small dose and gradually increasing the size of the dose. In some instances no appreciable tolerance can be established. A warning should be given that the "desensitizing dose," advised by some, may be large enough to kill a highly sensitive individual. How exquisitely hypersensitive some atopic individuals may be is shown by the fatality reported by Cooke.[1] In this instance death followed the intracutaneous injection of 0.02 c.c. of a glue solution containing 0.1 mg. of nitrogen per cubic centimeter.

Prevention of Serum Reactions.—It is usually recommended that at least those cases who give a history of asthma, hay-fever or other idio-

[1] Jour. Immunol., 1922, **7**, 119.

syncrasies due to hypersensitiveness, or those who have previously been injected with antitoxin or antiserum, be tested for the presence of hypersensitiveness by skin test. Evidence indicates that this is not a satisfactory criterion. As will be noted below, the injection of horse serum may induce a skin sensitiveness in man, but the injection of antitoxin in these cases may result in no general reaction. Tests carried out at the Willard Parker Hospital have shown no close correlation between skin reactions and reactions following the injection of antitoxin. This is in agreement with the results obtained by Cooke with pollen and other extracts. Cooke[1] believes that the conjunctival instillation of horse serum or antitoxic globulin is a much better criterion of the probability of systemic reaction. A drop of serum or antitoxin may be used for the test. Adrenalin should be available and used in the rare cases in which the reaction is severe. Mesereau and Cooke[2] recently had a case of diphtheria who was skin sensitive to horse proteins. Because of the indicated risk, fractional administration of diphtheria antitoxin in increasing sized doses was done. Each injection was followed by marked local skin reaction, but not the slightest sign of systemic reaction occurred nor later any serum sickness. Unfortunately the eye test was not done in this case, but the presumption is that the hypersensitiveness was not atopic. Coca believes that even the least sensitive cases of atopy cannot be safely given more than 0.1 c.c. of serum because of the severe symptoms which may result. There are two factors that should be considered before accepting a recommendation that a test for hypersensitiveness be always employed before serum is administered: (1) Expediency, that is, the extremely rare occurrence of death; (2) possible delay in the administration of an urgently needed remedy. It would seem that for practical purposes preliminary testing could be limited to those whose history of probable hay fever, asthma or food idiosyncrasy indicates that they will have severe reactions. With such a history and evidences of marked sensitiveness as revealed by test, the fractional administration starting with very small doses may be successful. Even in such cases it becomes a question of judgment as to which is the greater risk, delay in administration or the injection of a curative dose of serum. In such cases the risk is proportionate to the mode of injection, insofar as this influences the rate of absorption. Intravenous injection would be the most dangerous and intrathecal administration would also have to be feared. If the urgency for the remedy was not too great, one might attempt to induce some tolerance by preliminary subcutaneous injection. It is most important that intravenous or intrathecal introduction be very slow. As was stated previously, we do not know whether the deaths in cases of status lymphaticus were in any way due to an atopic condition. The presumption is that such cases are generally bad risks, that the slight shock of the parenterally introduced protein was sufficient to break down an existing defective physiological mechanism. In cases of status lymphaticus the

[1] Personal communication.
[2] Ibid.

only recommendation that can be made is that fractional doses be given, if not contraindicated by the urgent need of the remedy.

Active Sensitization in Man.—Hooker[1] has reported that the injection of toxin-antitoxin made by the old formula will confer a skin sensitiveness to horse serum in a certain proportion of individuals. However, the preliminary sensitivity test of 0.1 c.c. of a 1 per cent. horse serum was given before the injections. The new preparation of toxin-antitoxin contains only about one-thirtieth of the horse globulin in the old. The three injections of the new toxin-antitoxin would be equivalent to the injection of only about 0.0003 c.c. of horse serum. Park[2] has verified Hooker's results except insofar as he finds a lower incidence of apparently acquired skin sensitiveness. His results show that there is a rough parallelism between the amount injected and the subsequent skin sensitiveness. He could not prove that the reinjection of serum in these cases was more likely to cause severe reactions than in cases which had not been treated. Children receiving the newer toxin-anti-toxin (0.1 L+ mixture) which contains about one-thirtieth of the toxin-antitoxin used by Hooker and no preliminary test dose of serum showed only a moderate increase of skin sensitivity. Hooker's expressed fear that the apparently acquired skin sensitiveness was possibly an index that untoward results would follow the injection of antitoxin seems to have no basis. A comparison of 100 who had toxin-antitoxin and 100 who had not showed no appreciable differences in reaction after serum injections. Whatever slight sensitization develops gradually diminishes.

In spite of these observations it is believed to be wise to change and add goat antitoxin to the toxin as was suggested by Hooker. This is just as good and it cannot possibly sensitize those getting it to later injections of horse serum.

In the previous discussion examples of apparent sensitization in man have been noted; violent or even fatal reactions after repeated injections of antitoxin or serum, especially when given intraspinously or intravenously. The question arises are these to be considered as examples of anaphylaxis in man. There are reasons why they should not be accepted as such without further consideration. As was noted, the guinea-pig is easily sensitized, the rabbit less easily and the dog with some difficulty. No one has been able thus far to render monkeys definitely anaphylactic. This would seem to indicate that man is relatively refractory. We cannot exclude the possibility that a few individuals can be rendered anaphylactic. The observations of Hooker and Park on sensitization and the accelerated or immediate serum sickness after second injections, as well as the development of local necrosis, indicate that man can be sensitized in a certain sense. That there is changed response after the parenteral introduction of protein is seen in the fact that a second dose of antitoxin given after a week or ten days (prophylactic use) is excreted more rapidly than the first dose. We are inclined to the opinion that, although man may possibly be rendered anaphylactic

[1] Jour. Immunol., 1924, **9**, 7.
[2] Ibid., p. 17.

(as narrowly defined), direct proof that this occurs is still to be obtained. In this connection it should be emphasized again that, because of their greater frequency, the extremely infrequent fatal outcome, as a rule, follows primary injections and that the fatal dose may be small, as compared with dose necessary to kill an anaphylactic guinea-pig.

Drug Idiosyncrasy.—Peculiar individual susceptibility to various drugs is not uncommon. Although the drug may be toxic, the reactions obtained apparently have no relation to the toxicity. Mercury, salvarsan, iodides, quinine, morphine and the salicylates are some of the agents which cause such reactions.

The Bacteriophage of Twort[1] (d'Herelle.)—This phenomenon has been discussed in an earlier chapter. If the bacteriophage acts in the human body as it acts in the test-tube it would greatly influence infection. Claims have been advanced that this is the case. Thus, if the intestinal phage can adapt itself sufficiently rapidly to the infecting bacterium this should result in aborting the infection or at least limiting its progress. The presumption is, therefore, that the complete development of enteric disease is due to the lack of opposition by the phage or to the infecting bacterium's being resistant or refractory to phage action. D'Herelle believes that in epidemics the phage as well as the bacterium is transferred in the contagion. It has been claimed that the phage is consistently present in the feces of convalescent enteric disease cases. The belief that the phage is a considerable element in determining the severity and recovery from infection is not generally accepted. D'Herelle has used the phage therapeutically, and believes that it is of distinct value in the treatment of bacillary dysentery, in staphylococcus infections and possibly in typhoid fever and plague. The question has been raised whether the favorable clinical results may not be due to non-specific factors. Bronfenbrenner and others have failed to influence experimental infections. The indications are that the phage does not accumulate *in vivo* in amount comparable to that in the test-tube. Several factors apparently operate in this limitation, the slower multiplication, adsorption of the phage by the colloids of the body cells and fluids and the rapid elimination through the bile and urine.

[1] For fuller discussion, see d'Herelle.: Immunity in Natural Infectious Diseases; Bronfenbrenner: Newer Knowledge of Bacteriology and Immunology, edited by Jordan and Falk.

IDENTIFICATION[1] OF PATHOGENIC BACTERIA INCLUDING HIGHER BACTERIA AND SPIROCHETES ALSO CERTAIN NON-PATHOGENIC FORMS IMPORTANT IN DIAGNOSIS.

Names. Common with Chief Synonyms.	Recommended[2] by Committee of Society of American Bacteriologists.	Chief Identifiers[3] with Dates.		Endogenous	Flagella	Gram stain[4]	Free oxygen required	Indol formed	H_2S formed	Nitrates reduced	Gelatin liquefied	Milk: Coagulated	Milk: Peptonized	Carbohydrate fermentations[6]: Dextrose (glucose)	Lactose	Sucrose (saccharose)	Maltose	Mannitol (mannite)	Salicin	Inulin	Starch	Russell's lactrose-dextrose-mannite	Poisons[7]: Specific exotoxin	Hemolysin or other lysins	Leucocydin	Endotoxin	Antibodies[8]: Antitoxin	Agglutinins: Types	Agglutinins: Hetero-genous	Bacteriocidin	Opsonin	Pathogenicity, according to site: Skin and subcutis	Mouth and nasopharynx	Gastrointestinal tract and peritoneum	Respiratory tract and pleura	Eye	Names of lesions, and diseases produced indicated under sites (see key): Ear	Nervous system	Genital tract	Urinary tract	Circulatory system and pericardium	Lymphatic system	Bones and joints	Liver and pancreas
Orange staphylococcus	Staphylococcus[9] aureus	Pasteur, also Koch	1878	−	−	+	±	−	+	+	+	+	−	A	A	A	A	A	−	−	..	A	..	+	+	+	+	+	3?	+	+	+	e f g h i j t x y	A B D E F U P E W	a c d f h i j t x y	a b c d h j q r s t w x y	B R BN EM L PS	c e f q t w x	B L CJ K OP	c h t x y	M T OT	E N ME	a c d h i q r t x y	a c d h t q r t x y	C Y N P Y U R	p r	L Y P R	p r y w h	b c h t u	A H OS PO SY	h q r s v w y	C O H P
Staphylococcus pyogenes aureus	.....	Rosenbach	1884	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Golden staphylococcus	.....	Garré	1885	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
White staphylococcus	Staphylococcus albus	Rosenbach	1884	−	−	+	±	−	+	+ or −	+	+	−	A	A	A	A	A	−	−	..	A	..	±	±	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Staphylococcus pyogenes albus	.....			..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Staphylococcus of stitch abscess	Staphylococcus epidemitis	Welch	1891	−	−	+	±	−	+	+ or −	±	±		A	A	A	..	−	−	−	..	A	..	±	±	..	..	..	2?	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Lemon staphylococcus	Staphylococcus citreus	Passet	1885	−	−	+	±	−	+	+	+	+	−	A	A	A	A	A	−	−	..	A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Staphylococcus pyogenes citreus	.....			..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Micrococcus tetragenus	Gaffkya tetragenus	Gaffky	1881	−	−	+	±	−	−	−	−	±	−	A	A	..	..	..	..	..	..	A	..	..	..	..	..	..	..	..	..	..	..	i [10]	..	..	a c	BR	c	CJ	..	..	..	..	..	..	..	..
Hemolytic streptococcus	Streptococcus[11] pyogenes	Ogston	1881	−	−	+	±	−	..	..	−	+ or −	−	A	A	A	..	−	A	−	..	A	+?	+	+	+	+	6	+	+	+	b c d E R I W B I P E W	b c d f h i j t u	a c d h i s q t u a c	a b c d h i j t u	B R BN EM IN L PS	c d f h l t	B L CJ I R K OP OH	c d h t	M T OT	E N ME	c d h i j q r t	C E O E D M S O R P P S V	C Y N P Y U R	o r	A R E D PI PL PF SP	o r	L Y P R	b c d h t	A H OS PO SY	h q r s t	H P
Beta hemolytic streptococcus	.....	Fehleisen	1883	..	..	..	..	..	..	..	..	or	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Streptococcus hemolyticus	.....	Rosenbach	1884	..	..	..	..	..	..	..	..	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Streptococcus erysipelatis, scarlatinæ, etc.	.....	Schottmüller	1903	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Streptococcus hemolyticus V (Holman)	Streptococcus mixtas	Holman	1916	−	−	+	±	−	..	..	−	−	−	A	A	..	..	A	A	−	..	A	..	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Streptococcus infrequens (Holman)	.....			..	..	..	..	..	..	..	..	or −	..	..	or −	..	..	or −	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Streptococcus equi	Streptococcus equi	Schutz	1888	..	..	..	..	..	..	..	..	+	..	A	A	..	..	−	A	−	..	A	..	+	..	..	..	?	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Green[12] streptococcus	Streptococcus mitior	Schottmüller	1903	−	−	+	±	−	−	−	−	+	−	A	A	A	..	−	A	−	..	A	..	+	..	..	..	..	..	..	..	..	..	a T O c R H h S I	..	..	..	..	..	..	..	..	..	..	..	..	..
Alpha streptococcus	.....	Andrews & Horder	1906	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	−	..	..	..	..	..	..	+	..	+	a c	A P	..	..	B R BN EM	c h i	C J	c M T OT	q E N	a c	C E C U R	o c y	E D	o c	b c y	R E SY AH	
Streptococcus viridans	.....	Holman	1916	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Streptococcus mitis	.....			..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Streptococcus salivarius	Streptococcus salivarius	Andrews & Horder	1906	−	−	+	±	−	..	..	−	+	−	A	A	A	..	−	A	−	..	A	..	−	..	..	..	..	..	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Streptococcus fecalis	Streptococcus fecalis	Andrews & Horder	1906	−	−	+	±	−	..	..	−	+	−	A	A	A	..	A	A	−	..	A	..	−	..	..	..	..	..	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Streptococcus lactis (?)	Streptococcus lactis	Lister	1903																																												
Enterococcus (?)																																															
Gamma streptococcus	Streptococcus saprophyticus	Mandelbaum	1907	−	−	+	±	−	..	..	−	+	−	A	A	A	..	A	A	−	..	A	..	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Streptococcus anhemolyticus	.....	Zeitmeister	1910									or −			or −		or −																[10]													
Streptococcus saprophyticus																																														
Pneumococcus, Capsule coccus, Diplococcus or Micrococcus lanceolatus	Diplococcus[13] pneumoniæ	Sternberg	1880	−	−	+	±	−	..	..	−	−	−	A	A	A	..	−	..	A	..	A	+	+	+	+	+	?	2	+	+	+	h	a c h	P H S I T O	a b c d q t	A P E T G P T U L	b c d h q r s t w	B R BN EM IN L PS	c h i	C J I R O H O P	c	O T	b c d	M E	c h		c h q		n o	E D PI	n o		A H SY	q h
		Pasteur	1880	..	..	..	..	..	..	..	..	..	..	..	..	..	..	or	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
		Frankel	1884	..	..	..	..	..	..	..	..	..	..	..	..	..	..	A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
		Weichselbaum	1886	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Pneumococcus mucosus	Diplococcus pneumoniæ	Park & Williams	1905	−	−	+	±	−	−	−	−	+	−	A	A	A	..	A	..	A	..	A	?	−	+	+	?	1	..	+	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Diplococcus mucosus	Type III			..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Streptococcus mucosus	.....			..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Meningococcus, Micrococcus or Diplococcus intracellularis meningitides	Neisseria intracellularis	Weischselbaum	1887	−	−	−	±	..	..	−	−	−	−	A	−	−	A	−	-	−	−	..	..	−	..	+	..	4	+	¬	¬	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Micrococcus catarrhalis	Neisseria catarrhalis	Pfeiffer	1896	−	−	−	±	..	..	−	−	−	−	−	−	−	−	−	−	−	−	..	..	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Diplococcus siccus	Neisseria sicca[14]	v. Lingelsheim	1908	−	−	−	±	..	..	−	−	−	−	A	−	A	−	−	−	−	−	..	..	−	..	..	..	..	..	..	..	..	..	a c	P H R H	[10]	a c	B R	[10]	..	..	..	..	..	..	..	..	..
Gonococcus	Neisseria gonorrhœa	Neisser	1879	..	..	..	..	..	..	..	..	..	..	A	−	−	−	−	−	−	−	..	..	−	..	+	..	11	+	+	+	..	..	b c	G O P T	..	c q t	G O	..	..	..	c h i t	G O	c h o	G O	o	b c	G O	..
Micrococcus gonorrhœa	.....	Bumm	1885	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Diphtheria bacillus	Corynebacterium diphtheriæ[15]	Klebs	1883	−	−	±	±	−	−	−	−	−	−	A	−	−	A or −	−	−	..	..	..	H	−	..	?	H	8	+	?	..	b c	D P	b c t	D P	q	..	b c t	D P	b c t	D P	b c	D P	q s	..	q s	D P	q r s	D P	n r s	..	n r s	..	q r
Klebs-Loffler bacillus	.....	Löffler	1884	..	..	..	..	..	..	..	..	..	..	..	..	..	or	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Bacillus diphtheriæ	.....	Roux-Yersin	1888	..	..	..	..	..	..	..	..	..	..	..	..	..	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Pseudo diphtheria bacillus	Corynebacterium hofmanni	Hofmann	1888	−	−	¬	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Bacillus hofmanni																																																
Bacillus xerosis	Corynebacterium xerosis	Neisser & Kutschid	1884	−	−	+	±	−	..	−	−	−	−	A	−	A	A	..	..	..	..	..	..	..	..	..	?	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
																																									a c	X [10]								
Bacillus segmentosus	Corynebacterium segmentosum	Cautley	1894	..	..	..	..	..	..	..	..	..	..	A	A	A	..	..	..	..	..	..	..	..	..	?	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
																																a c	R H [19]														
Bacillus hodgkinii	Corynebacterium hodgkinii	Bunting & Yates	1914	−	−	+	±	..	..	¬	−	−	−	A	A	A	A	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Bacillus acne	Corynebacterium acne	Gilchrist	1901	−	−	¬	±	..	..	−	−	−	−	A	..	..	..	..	..	..	..	..	..	..	..	..	?	..	..	..	?	..	c A f	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Lymph node anaërobic bacillus	Corynebacterium lymphophylum	Torrey	1916	−	−	−	−	..	..	..	..	−	..	A	−	−	− or A	..	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Vincent's bacillus, Bacillus fusiformis	Fusiformis dentium	Babes	1889	−	−	∓	−	+	..	..	−	−	−	A	−	−	A or −	−	−	−	−	..	..	..	..	..	..	..	..	..	..	..	..	..	d u	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Colon bacillus	Escherichia[17] coli	Escherich	1886	−	+	−	±	+	..	+	−	+	−	AG	AG	−	AG	AG	AG	..	..	A	AG	..	..	..	+	..	..	¬	¬	+	l [16]	..	a b c d h t [16]	AP DI PT	a b c d h t [16]	B R BN	c	C J	c	O T	b c q	M E	a b c d h t	C E EO V	a b c d h r t	C Y N P Y	o	E D PI [16]	o		b c	R E	c q s t v [16]	C O H P
Bacillus coli communis	.....			..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Bacillus coli communior	Escherichia communior	Durham	1900	−	+	−	±	+	..	+	−	+	−	AG	AG	AG	AG	AG	AG	..	..	A	AG	..	..	..	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Bacillus acidi lactici	Escherichia acidi-lactici	Hueppe	1884	−	−	−	±	−	..	+	−	+	−	AG	AG	−	AG	AG	−	..	..	A	AG	..	..	..	?	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Bacillus lactis ærogenes	Aerobacter ærogenes	Escherich	1886	..	−	−	±	+	..	..	+	−	+	AG	AG	AG	AG	AG	..	..	..	A	AG	..	..	..	?	..	..	..	..	..	..	[16]	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Pneumobacillus	Klebsiella pneumoniæ	Friedlander	1882	−	−	−	±	−	..	..	+	−	+	AG	AG	AG	..	..	..	..	..	A	AG	..	..	..	?	..	..	..	..	−	..	..	[10]	..	a b c	BR BN	..	..	..	..	..	..	..	..	o	..	..	..	..
Bacillus mucosus capsulatus	.....			..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Bacillus of rhinoscleroma	Klebsiella rhinoscleromatis	v. Frisch	1882	−	−	−	±	−	..	+	−	+	−	−	−	..	..	..	..	..	..	−	−	..	..	..	..	..	..	..	..	..	..	..	y R I [10]	..	..	..	..	..	..	..	..	..	..	..	..	..	..
												AG	AG							A	AG																										
Bacillus ozenæ	Klebsiella ozenæ	Abel	1893	−	−	−	±	−	..	−	−	−	−	−	−	..	..	..	..	..	..	−	−	..	..	..	..	..	..	..	..	..	..	..	a O Z c [19]	..	..	..	..	..	..	..	..	..	..	..	..	..	..
												AG	AG							A	AG																										
Typhoid bacillus	Eberthella[17] typhi	Eberth	1880	−	+	−	+	−	..	+	−	−	−	A	−	−	A	A	..	..	..	−	A	..	..	..	+	..	1	..	+	+	e	y	y i e a c	TY DY	q			q		q	q	q r	o y	o y w	L Y P R		q y
Bacillus typhosus	.....	Gaffky	1884	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Dysentery bacillus (Bacillus dysenteriæ)	Eberthella dysenteriæ (Shiga)	Shiga	1898	−	−	−	±	−	..	−	−	−	−	A	−	−	−	−	..	..	−	A	..	..	..	+	±	1	..	+	..																
Dysentery bacillus (Flexner)	Eberthella paradysenteriæ (Flexner)	Martin & Lentz	1902	..	−	±	..	−	−	−	−	A	−	A	−	A	..	..	−	A	..	..	..	+	..	1	..	+																		
Paradysentery bacillus	.....	Park	1903																																												
		Hiss	1905																																												
Paradysentery bacillus (Park type)	Eberthella paradysenteriæ (Park)	Park	1903											A		−	−	A			−	A				+	..	1	..	+																
		Hiss	1905																																												
Dysentery bacillus (Harris-Wollstein type)	Eberthella paradysenteriæ (Wollstein)	Wollstein	1903											¬		A	A	A				¬				+	..	..	..	+																
Paradysentery bacillus	.....	Hiss	1904																																											
Paratyphoid bacillus A	Salmonella[17] paratyphi	Widal	1897	−	+	−	±	−	..	−	−	−	−	AG	−	−	AG	AG	..	..	..	−	A	..	..	..	+	..	1	..	−				y i	PA								o y	o y			
		Gwyn	1898																																											
		Schottmüller	1900																																												
		Brion & Kayser	1902																																											
Paratyphoid bacillus B	Salmonella schottmülleri	Schottmüller	1900	−	+	−	±	−	..	−	−	−	−	AG	−	−	AG	AG	−	..	−	AG	..	..	..	+	..	1	..	−																
		Brion & Kayser	1902																																											
Bacillus enteritidis	Salmonella enteritidis	Gaertner	1888											AG	−	−	AG	AG	..	..	−	AG	..	..	..	−	..	..	+	..	..			a c	FO												
Bacterium fœtidum, Perez bacillus	Salmonella fœtida	Perez	1899											AG	−	..	..	−	..	..	−	AG												a RH y												
Bacillus alkaligenes	Alkaligenes fecalis	Petruschky	1896	−	+	−	±	−	..	+	−	−	−	−	−	−	−	−	−	−	−	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Bacillus bronchisepticus	Bruecella bronchisepticus	Ferry	1909	−	¬	−	±	−	..	−	−	−	−	−	−	−	−	−	−	−	−	−	..	..	..	..	+	..	..	..	..	..	..	..	[18]	..	..	..	..	..	..	..	..	..	..	..	..	..
Bacillus abortus	Brucella abortus[13]	Bang	1897	−	−	−	±	−	..	−	−	−	−	−	−	−	−	−	−	−	−	−	..	..	..	..	+	..	..	..	..	..	..	..	[18]	..	..	..	..	..	..	..	c	o	o	..	..	..	..
Bacterium of malta fever	Brucella melitensis	Bruce	1887	−	−	−	±	−	..	−	−	−	−	−	−	−	−	−	−	−	−	−	..	..	..	..	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	q	..	..	..
Bacterium or micrococcus melitensis	.....			..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Bacillus proteus vulgaris	Proteus[13] vulgaris	Hauser	1885	−	¬	−	±	+	..	¬	+	−	..	AG	−	AG	AG	−	..	..	−	AG	..	..	..	?	..	..	..	..	..	..	..	..	b c	P O P A	b c	P S	c	C J	c	O T	..	..	c	E O	c	C Y						
Bacillus proteus mirabilis	Proteus mirabilis	Hauser	1885	..	+	−	±	..	..	¬	+	..	..	AG	−	AG	−	−	..	..	−	AG	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	o	..	o	..	..
Bacillus valeriei	Proteus valeriei	Boycott	1906	−	+	−	±	¬	..	¬	−	−	−	AG	−	AG	AG	AG	AG	..	−	AG	..	..	..	..	?	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	y	..	..	..	..
Bacillus hydrophilum fuscus	Proteus hydrophilis[20]	Sanarelli	1891	−	+	−	±	¬	..	+	+	+	−	AG	A	AG	..	AG	..	..	..	A	AG	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	o [20]	..	o	..	..	d
Bacillus pyocyaneus	Pseudomonas æruginosa[21]	Schroter	1872	−	¬		±	¬		¬	+	+	+	..	A	..	..	..	..	..	¬	..	¬	?																													
		Gessard	1882																																																	
Tubercle bacillus	Mycobacterium[22] tuberculosis (hominis)	Koch	1884		−	¬	¬																																												
Bacillus tuberculosis, human type																																																			
Bovine tubercle bacillus	Mycobacterium tuberculosis (bovis)	Th. Smith	1896		−	+	¬			−																																								
Bird tubercle bacillus	Mycobacterium avium	Strauss & Gamalia	1891	−		¬	+																																										
Johne's bacillus, Serapic bacillus	Mycobacterium paratuberculosis	Johne	1895		−	+	¬			−																																						
Smegma bacillus	Mycobacterium smegmatis	Alvares & Tavel	1885	−	−	+	¬			−																																						
Turtle acid fast bacillus	Mycobacterium chelonai	Friedmann	1903	−	−	+	+			−																																						
Butter acid fast bacillus	Mycobacterium berolinensis	Rabinowitsch	1897	−	−	+	+																																								
Leprosy bacillus	Mycobacterium lepræ[23]	Hansen	1872	−	−	¬																																									

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KEY TO NAMES OF CHIEF LESIONS[1] AND DISEASES[2] PRODUCED BY BACTERIA.

Names of Chief Lesions (Indicated in the Table by the Small Letters Before Them).

A. Inflammations[3]

I. *Named according to exudate*
- a Mucous (catarrhal)
- b Serous or sero-fibrinous } Croupous, diphtheritic or pseudomembranous
- c Purulent (suppurative) }
- d Hemorrhagic

II. *Named according to local manifestations*
- e Macular, may become *papular*
- f Vesicular, usually becomes *pustular*
- g Furuncular, may result in *carbuncle*
- h Abscess } Usually produce more or less serous and purulent edema and may lead to fistular, sinus or cyst
- i Ulcer }
- j Phlegmon }
- k Chancre—hard
- l Bubo
- m Gumma

III. *Named according to conveyance through blood and lymph channels*
- n Toxemia
- o Septicemia } Bacteriemia, may produce septic thrombus
- p Pyemia }

B. Degenerations

- q Albuminous (parenchymatous, granular, cloudy swelling)
- r Hyaline
- s Fatty
- t Necrotic, including coagulation necrosis, focal necrosis, cheesy, etc.
- u Gangrenous, including gasseous gangrene

C. Infiltrations

- v Amyloid
- w Pigment (from blood, bile, etc.)

D. Reparative Changes

- x Granulation tissue
- y Hyperplasia (productive inflammation), may lead to production of interstitial tissue and cicatrices

[1] Lesions may vary within limits, in type, intensity and site, according to character of organism (including virulence under which may be placed numbers and concentration), resistance of host, period of disease, whether primary, mixed or secondary infections. They usually merge into each other and form very complex pictures, *e. g.*, mucous inflammation may quickly become mucopurulent. A number of specially named lesions have not been included. The primary point of entry must of course be through mucous membranes or skin and is probably always the result of an injury however slight. The lesions are indicated as far as possible according to course and intensity.

[2] Not all diseases named from symptoms or lesions are included. The definite relations between disease, and lesions are not shown except with those organisms producing a specific disease. In these cases the capital letters indicating the disease are placed only in the column or columns containing the most characteristic lesions. The list of diseases given here are supposed to be due in part at least, to bacteria. Some are due to mixtures, many are complicated by secondary infections.

[3] Inflammation is the vascular and cellular response of an individual to an injury. It is a defensive process, therefore when possible it merges quickly into repair.

Names of Chief Diseases Accepted as Due to Bacteria (Indicated in Table by the Capital Letters Before Them).

- A Acne
- AC Actinomycosis (nocardia, streptothricosis)
- AN Angina, Ludwig's
- AM Angina, membranous
- AV Angina, Vincent's
- AT Anthrax (splenic fever) malignant fever)
- AP Appendicitis
- AR Arteritis
- AH Arthritis
- B Blackleg (symptomatic anthrax)
- BI Boil
- BL Blenorrhœa
- BO Botulism in humans (limber neck in chickens)
- BR Bronchitis
- BN Bronchopneumonia (lobular pneumonia) Carbuncle (lesion g)
- C Cellulitis
- CE Cervicitis
- CH Chancroid (soft chancre)
- CO Cholecystitis
- CL Cholera
- CJ Conjunctivitis
- CU Conjunctivitis, acute contagious
- CY Cystitis
- D Dermatitis
- DI Diarrhœa
- DP Diphtheria
- DY Dysentery (bacillary)
- E Eczema
- EA Edema, malignant
- EM Empyema
- EN Encephalitis
- ED Endocarditis
- EO Endometritis
- ET Enteritis
- EP Epididymitis
- ER Erysipelas
- F Farcy
- FO Food poisoning (paratyphoid group, etc.)
- FU Furunculosis
- G Gastritis (phlegmonous, etc.)
- GL Glanders
- GO Gonorrhœa (or gonococcus infections)
- H Hepatitis
- HE Hemorrhagic septicemia
- I Impetigo
- IN Influenza, non-epidemic
- IR Iritis
- J Jaundice, epidemic or infective
- K Keratitis
- L Laryngitis
- LE Leprosy Lockjaw (see tetanus)
- LO Lobar pneumonia
- LU Lupus vulgaris
- LY Lymphangitis
- M Madura foot (mycetoma) Malignant edema (see edema)
- MA Malta fever
- MS Mastitis
- MT Mastoiditis
- ME Meningitis
- MN Meningitis, acute contagious
- N Nephritis Nocardia (see actinomycosis)
- O Oöphoritis
- OP Ophthalmia
- OH Ophthalmia neonatorum
- OR Orchitis
- OS Osteomyelitis
- OT Otitis media
- OZ Ozena
- P Pancreatitis
- PA Paratyphoid fever
- PR Parotitis
- PE Pemphagus
- PI Pericarditis
- PO Periostitis
- PT Peritonitis
- PU Pertussis (whooping-cough)
- PH Pharyngitis
- PL Phlebitis
- PG Plague
- PC Plague or septicemia of lower animals
- PS Pleurisy Pneumonia (see broncho and lobar)
- PP Prostatitis
- PF Puerpural fever
- PY Pyelitis
- R Relapsing fever (tick fever)
- RH Rhinitis
- RE Rheumatism
- RI Rhinoscleroma
- S Salpingitis
- SE Septic sore throat
- SP Septic thrombus
- SY Seven-day fever
- SI Sinusitis Soft chancre (see chancroid) Streptothricosis (see actinomycosis)
- SY Synovitis
- SH Syphilis
- T Tetanus
- TO Tonsillitis
- TU Tuberculosis
- TL Tularæmia
- TY Typhoid fever
- UL Ulcer of stomach
- UR Urethritis
- V Vaginitis Vincent's angina (see angina, etc.)
- W War wounds (gaseous gangrene, etc.) Whooping-cough (see pertussis.) Woolsorters' disease (see anthrax)
- X Xerosis
- Y Yaws
- YE Yellow fever

PART II.

PATHOGENIC MICROÖRGANISMS INDIVIDUALLY CONSIDERED.

CHAPTER XV.

THE PYOGENIC COCCI.

Many species of microörganisms, when inoculated into the animal body, have been shown by experiment to induce, under certain conditions, the formation of pus by their products. But, practically, only a few species are concerned in the production of acute abscesses in man. Of these the two most important, by reason of their frequent occurrence and pathogenic power, are Staphylococcus aureus and Streptococcus pyogenes. These two organisms are often found in the same abscess; thus, Passet, in 33 cases of acute abscess, found Staphylococcus aureus alone in 6, aureus and albus associated in 11, albus alone in 4, albus and citreus in 2, Streptococcus pyogenes alone in 8, albus and Streptococcus in 1, and albus, citreus, and Streptococcus in 1. The staphylococcus is likely to enter as a mixed infection into many infections due to other bacteria, and is almost always met with in inflammations of the skin and mucous membranes or in cavities connected with them. A large group of saprophytic cocci found in water, soil and air form the second genus of the tribe Micrococceæ, one of the three tribes of the coccus family (Coccaceæ). The first genus of this tribe is Staphylococcus. The genera Sarcina and Rhodococcus, the species of which are probably all saprophytic, are placed in the same tribe.[1]

THE STAPHYLOCOCCI.

Staphylococci were first obtained from pus by Pasteur in 1878. In 1881 Ogston[2] showed that they frequently occurred in abscesses, and in 1884 Rosenbach[3] fully demonstrated their etiological importance in circumscribed suppurative inflammations, osteomyelitis, etc. Of the staphylococci those producing yellow and white pigments are by far the most important since they are the pathogenic varieties.

[1] Bergey's Manual of Determinative Bacteriology, Baltimore, 1925.
[2] British Med. Jour., 1881, 7, 369.
[3] Microörganismen bei Wundinfektion, Wiesbaden, 1884.

The Staphylococcus (Pyogenes) Aureus.—The *Staphylococcus aureus* is one of the commonest parasitic bacteria, being usually present in the skin and mucous membranes, and is the organism most frequently concerned in the production of acute, suppurative inflammations.

Morphology.—Small, spherical cells, having an average diameter of 0.7 μ to 0.9 μ occurring solitary, in pairs as diplococci, in short, loose, uneven rows of three or four elements, or in groups of four, but most commonly in irregular masses, simulating clusters of grapes; hence the name staphylococcus. (See Fig. 95.)

Staining.—It stains quickly in aqueous solutions of the basic anilin colors and is not decolorized by Gram's method. When slightly stained each sphere frequently is seen to be dividing into two hemispherical bodies.

Biology.—The Staphylococcus aureus is a non-motile, aërobic, facultative anaërobic micrococcus, growing at a temperature from 8° to 43° C., but best at 25° to 35° C. It grows readily on all the common laboratory media, best in a slightly alkaline medium, but they also grow in slightly acid media.

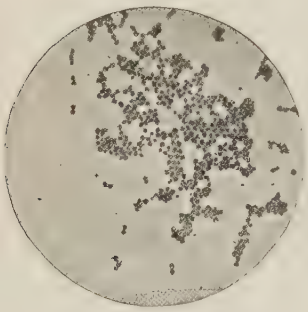


FIG. 95.—Staphylococcus. × 1100 diameters.

Cultivation.—*Growth in Nutrient Bouillon.*—The growth of the staphylococcus is rapid, reaching about 500,000,000 per cubic centimeter at the end of twenty-four hours at 30° C. The bouillon is cloudy and frequently has a thin pellicle, which may adhere slightly to the test-tube. Later a slimy sediment forms. The odor is disagreeable. Grown in peptone water, no indol is in evidence.

Growth on Gelatin.—Grown on gelatin plates at room temperature, within forty-eight hours punctiform colonies develop, which, when examined under a low-power lens, appear as circular disks of a pale brown color, somewhat darker in the center, and surrounded by a smooth border. The macroscopic appearance of the growth is characteristic. Surrounding the colonies, which are of a pale golden-yellow color, there is a pitting of the surface of the gelatin, due to its liquefaction. By suitable light these shallow depressions with sharply defined outlines may be seen, having a diameter of from 5 to 10 mm., in the centers of which lie the yellow colonies. Later the liquefaction becomes general. In stab cultures in gelatin a white confluent growth at first appears along the line of puncture, followed by a funnel-shaped liquefaction of the medium, which rapidly extends to the sides of the test-tube. At the end of two days the yellow pigmentation begins to form, and this increases in intensity for eight days. Finally, the gelatin is completely liquefied, and the staphylococci form a golden-yellow or orange-colored deposit at the bottom of the tube. Under unfavorable conditions the Staphylococcus aureus gradually loses its

ability to make pigment and to liquefy gelatin. The liquefaction is due to a ferment called gelatinase formed by the staphylococci. It may be separated from the cocci by filtration (Loeb[1]).

Growth in Agar.—In streak and stab cultures on agar a whitish growth is at first produced, and this at the end of a few days becomes a faint to a rich golden-yellow on the surface. The yellow pigmentation is produced only in the presence of oxygen; colonies formed at the bottom of a stab culture or under a layer of oil remain white.

Milk.—Milk is coagulated at the end of from one to eight days.

Potato.—The staphylococci grow readily on potato and produce abundant deep-colored pigment.

Growth on Löffler's Solidified Blood Serum.—Growth vigorous, with fairly good pigment production. Some varieties slowly liquefy the serum.

Growth on Blood Agar.—If nutrient agar to which a little animal blood has been added is streaked with certain strains of staphylococci, there appears, at the end of twenty-four hours at 35° C., about the growth a clear zone, owing to the hemolytic effect of the staphylococcus products.

Acids Produced.—In media containing carbohydrates there is, as a result of the growth of the Staphylococcus aureus, a production of acid in considerable quantities, consisting chiefly of lactic, butyric, and valerianic acids. These acids have been supposed to play a part in the production of pus, in which, according to some observers, they are often present. No gas is formed.

Resistance.—The staphylococcus is distinguished from most other non-spore-bearing pathogenic bacteria by its greater power of resistance to outside influences, desiccation, etc., as well as to chemical disinfectants. Cultures of the Staphylococcus pyogenes in gelatin or agar retain their vitality for a year or more. For thermal death point and resistance to chemicals, see Disinfection. Cold has but little effect. Thirty per cent. of the organisms remained alive after being subjected by us to freezing in liquid air for thirty minutes. These are average figures. Some cultures are more resistant than others. They are quite resistant to direct sunlight and drying. Dried pus contains living staphylococci for weeks and even months, and they can be found alive in the fine dust of the air in living rooms and operating rooms.

Pigment Formation.—Pigment formation is considered within limits a species characteristic. Thus different strains of Staphylococcus aureus produce a pigment varying from a pale brown to a deep golden-yellow. It usually becomes less intense upon prolonged cultivation. The pigment is classed as a lipochrome (Schneider[2]). It is excreted from the organism but remains attached to the cell because it is insoluble in a watery medium. It is soluble in alcohol, chloroform and ether.

In order to test the amount of color produced, Winslow and Rogers recommend the following method: A portion of the growth is removed on a loop needle and spread out on white drawing paper with a rough surface. After drying at room temperature the color is compared with a standard color chart.

[1] Centralbl. f. Bakt., 1902. **32**. 471.
[2] Arb. a. d. bakt. Inst. Karksruhe, 1891, vol. **2**.

Pathogenesis.—The pathogenic effect of the Staphylococcus aureus on test animals varies considerably, according to the mode of application, the virulence of the special culture employed and the species of animal used. While in man a simple rubbing of the surface of the unbroken skin with pus from an acute abscess is often sufficient to produce a purulent inflammation,[1] and the introduction of a few germs from a septic case into a wound may lead to a fatal pyemia, in the lower animals these conditions can only be reproduced with difficulty, and by the inoculation of large quantities of the culture. Small subcutaneous injections, or the inoculation of open wounds in mice, guinea-pigs, and rabbits, are commonly without result; occasionally abscess formation may follow at the point of inoculation, which usually ends in recovery. Slightly virulent cultures, which constitute the majority of those obtained from pus taken from the human subject, when injected subcutaneously in large quantities (several cubic centimeters of a fresh bouillon culture) into rabbits or guinea-pigs, give rise to local pathological lesions—acute abscesses. When virulent cultures are used—the few among those recently isolated from human infections—0.5 c.c. of a fresh bouillon culture is sufficient to produce similar results. The abscesses generally heal without treatment; but sometimes the animals die from marasmus in consequence of the suppurative process. In intraperitoneal injections the degree of virulence of the culture employed is still more evident in the effects produced. The animals may die in from two to nine days. The most characteristic pathological lesions are found in the kidneys, which contain numerous small collections of pus, and under the microscope present the appearances resulting from embolic nephritis. Many of the capillaries and some of the smaller arteries of the cortex are plugged up with thrombi, consisting of micrococci. Metastatic abscesses may also be observed in the joints and muscles. The micrococci may be recovered in pure cultures from the blood and the various organs; but they are not numerous in the blood and are often difficult to demonstrate microscopically. Intravenous injections of animals are followed by similar pathological changes. Orth and Wyssokowitch first pointed out that injection of staphylococci into the circulation of rabbits, whose cardiac valves have previously been injured, produced ulcerative endocarditis. It has been further shown by Ribbert that the same result may be obtained without previous injury to the valves by injecting into a vein the staphylococcus from a potato culture suspended in water. In his experiments not only the micrococci from the surface, but the superficial layer of the potato were scraped off with a sterilized knife and mixed with distilled water and the successful result is ascribed to the fact that the little agglomerations of micrococci and infected fragments of potato attach themselves to the margins of the valves more readily than isolated cocci would do. Not infrequently, also, in intravenous injections of young animals there occurs a localization of the injected material in the marrow of the

[1] Garré-Beit: Ztschr. klin. Chir., 1893, vol. 10.

small bones. This may take place in full-grown animals when the bones have been injured or fractured. The experimental osteomyelitis thus produced has been demonstrated to be anatomically analogous to this disease in man.

An increase in virulence of certain strains may be obtained by successive passage through susceptible animals.

Spontaneous infections of lower animals with staphylococci have been reported, notably among sheep, cattle and horses. A disease of the house fly is said[1] to be due to a staphylococcus.

Toxic Substances Produced.—Filtrates of cultures may contain toxic substances. Injected into the peritoneal cavity they may excite peritonitis. Under the skin they may produce infiltration or abscess formation. Into the skin they cause a circumscribed red area of inflammation. This poison was shown by Parker[2] to be an exotoxin capable of producing an antitoxin. In the blood they may injure both the red and white corpuscles. The poison injuring the red blood cells is a lysin known as *staphylolysin* (Neisser and Wechsberg). It can be detected in cultures about the third and fourth day of incubation and reaches its maximum on the ninth to fourteenth day. Virulent staphylococci are apt to produce more of this substance than the non-virulent, but there is no definite rule. Julianelle[3] says that it seems to be associated with proteolysis.

This hemolysin is destroyed by heating for twenty minutes at 56° C. An antibody for this has been formed by inoculating animals with culture filtrates. The poison which injures leukocytes is called *leukocidin*. It also stimulates the production of an antibody.

Opsonin stimulating substances have also been demonstrated.

Transmissible Lytic Substance (*Bacteriophage*).—The first autolytic transmissible substance was obtained by Twort (see p. 305) for a staphylococcus. D'Herelle[4] obtained one from the pus of an infected finger. DeGracia obtained one from several samples of vaccine virus cultures.[5] Callow[6] made a study of staphylococcus bacteriophage and obtained such a substance in the pus of a series of sixteen staphylococcus infections.

Endotoxins.—Cultures of the staphylococcus, when sterilized by heat and injected subcutaneously, produce marked positive chemotaxis and often local abscesses. Leber found also that sterilized cultures introduced into the anterior chamber of the rabbit's eye would bring about a fibropurulent inflammation. These local changes follow the inoculation of small quantities only of the dead cultures; but when large amounts are injected into a vein or into the abdominal cavity, toxic effects are produced.

[1] Gaston: Am. Jour. Hyg., 1924, **4**, 411.
[2] Jour. Exp. Biol., 1924, **40**, 761.
[3] Jour. Infect. Dis., 1922, **31**, 256.
[4] Le Bacteriophage, monographies de l'Inst. Pasteur, 1921.
[5] Proc. Soc. Exp. Biol. and Med., 1920–21, **18**, 217.
[6] Jour. Infect. Dis., 1922, **30**, 644.

Occurrence in Man.—The staphylococcus (*Staphylococcus aureus*) has been demonstrated not only in furuncles and carbuncles, but also in various pustular infections of the skin and mucous membranes—impetigo, sycosis, purulent conjunctivitis and inflammation of the lacrimal sac; in acute abscesses formed in the lymphatic glands, the parotid gland, the tonsils, the mammæ, etc.; in metastatic abscesses and purulent collections in the joints; in empyema, infectious osteomyelitis, ulcerative endocarditis, pyelonephritis, abscess of the liver, phlebitis, meningitis, etc. It is one of the chief etiological factors in the production of so-called pyemia. It is remarkable how many staphylococci may be present in the blood without a fatal result, if the source of infection is removed. We met with one case in which over 800 staphylococci were present in 1 c.c. of blood. A week later only 5 were found. The blood finally became sterile.

A number of cases of staphylococcus pneumonia have been described as occurring among the troops during the World War.[1] There is always a possibility that the original infection was missed.

Not all persons are equally susceptible to infection by the staphylococcus; those who are in a cachectic condition or suffering from constitutional diseases, like diabetes, are especially predisposed to infection. In healthy individuals certain parts of the body, as the back of the neck and the buttocks, are more liable to be attacked than others, with the production of furuncles, carbuncles, etc. In persons in whom sores are readily caused, in consequence of disturbances of nutrition, as in exhausting diseases, the micrococci settle at the points of least resistance. Such conditions are present in the bones of debilitated young children, in fractures, and in injuries in general.

Immunity.—Rabbits have been rendered immune by means of inoculations with either dead or living cultures. Unless the inoculations are carefully made the animals frequently succumb. The staphylococci injected into an immunized animal are more rapidly taken up by the leukocytes than when injected into an untreated animal. (See Opsonins). A moderate but sometimes striking immunity in man is often developed following the use of vaccines. It lasts probably six to twelve months. Most people have some natural immunity.

A serum having some protective power has also been elaborated.

Hiss claimed good results from the use of leukocyte extracts in animals infected with *Staphylococcus aureus*.

Agglutinins have been produced in test animals, but the question of specific agglutinative types is not settled. Those who claim to have demonstrated specific types have not reported complete absorption tests.[2]

Therapeutic Use of Vaccine.—The best results have been obtained in chronic or recurrent types of infection, such as acne and furunculosis. Stock vaccines may be employed, but if failure is encountered autogenous vaccines should be tried. In the deep indurated types of acne other bacteria are probably important, as B. acne, and vaccines of the bacillus

[1] Chickering and Park: Jour. Am. Med. Assn., 1919, **72**, 617.

[2] Hine: Lancet, 1922, **2**, 1380; Julianelle: Jour. Infect. Dis., 1922, **31**, 256.

in doses of 2,000,000 to 20,000,000 should be tried with the staphylococcus. In furunculosis the vaccine seems to be of more value in preventing new lesions than in the cure of the existing foci. Sycosis and other skin lesions with associated pustular lesions may be benefited by vaccines. Acute local lesions are probably little influenced, and in this and in the other conditions mentioned the usual modes of treatment should be employed. The greatest care should be taken to protect the healthy skin from discharges from the infected focus.

Dosage.—The more extensive the lesions the smaller should be the initial dose. From 100,000,000 to 1,000,000,000 is the average increase in dosage, though larger doses may be given.

Staphylococcus Albus.—It is morphologically identical with the *Staphylococcus (pyogenes) aureus,* and may be the same organism which has lost the property of producing pigment. On the average it is somewhat less pathogenic and seldom produces pyemia or grave infections. The surface cultures upon nutrient agar and potato have a milk-white color. Its physiological requirements are the same as those of the *Staphylococcus aureus.*

The majority of bacteriologists agree with Rosenbach,[1] that the aureus is found at least twice as frequently in human pathological processes as the albus.

Staphylococcus Epidermidis (Welch).—According to Welch, this coccus differs from the *Staphylococcus albus* in the fact that it liquefies gelatin more slowly, does not so quickly cause coagulation in milk, and is far less virulent when injected into the circulation of rabbits. It has been shown by the experiments of Bossowski and of Welch that this microörganism is very frequently present in clean wounds, and that usually it does not materially interfere with the healing process, although sometimes it appears to cause suppuration along the drainage tube, and is the common cause of "stitch abscesses."

Staphylococcus (Pyogenes) Citreus.—Isolated by Passet (1885) from the pus of acute abscesses, in which it is occasionally found in association with other pyogenic cocci. It is distinguished from the other species only by the formation of a lemon-yellow pigment.

Other Staphylococci.—Other varieties have been occasionally met with which differ in some respects from the typical varieties. This difference may be in the fact that they liquefy gelatin more slowly or not at all, or in pigment formation, or in agglutination, or in still other respects. None of these varieties seem to be of importance.

THE MICROCOCCUS (GAFFKYA) TETRAGENUS.

This organism was discovered by Gaffky (1881) in purulent tuberculous sputum. It is not infrequently present in the saliva of healthy individuals and in the sputum of consumptive patients. In sputum it is more frequently an evidence of mouth contamination than of lung infection. It has been observed repeatedly in the walls of cavities in

[1] Microörganismen bei Wundenfektion, Wiesbaden, 1884.

pulmonary tuberculosis associated with other pathogenic bacteria, which, though playing no part in the etiology of the original disease, contribute, doubtless, to the progressive destruction of the lung. Its pyogenic character is shown by its occasional occurrence in the pus of acute abscesses. Its presence has also been noted in the pus of empyema following pneumonia.

Morphology.—Micrococci having a diameter of about 1μ, which divide in two planes, forming tetrads, and bound together by a transparent, gelatinous substance, enclosing the cell like a capsule. In cultures the cocci are seen in various stages of division as large, round cells in pairs of oval elements, and in groups of three and four (Figs. 96 and 97). When the division is complete they remind one of sarcinæ in appearance, except that they do not divide in three directions and are not built up like diminutive cotton bales (see also Plate I, Fig. 4, and Plate II, Fig. 11).

Staining.—This micrococcus *stains* readily with the ordinary anilin dyes; the transparent gelatinous envelope is only feebly stained. It is not decolorized by Gram's method.

Biology.—The growth of this micrococcus is slow under all conditions. It grows both in the presence and absence of oxygen; it grows best from 35° to 38° C., but may be cultivated also at the ordinary room temperature—about 20° C.

Growth on Gelatin.—On gelatin plates small, white colonies are developed in from twenty-four to forty-eight hours, which, when examined under a low-power lens, are seen to be spherical or lemon-shaped, grayish-yellow disks, with a finely granular or mulberry-like surface, and a uniform but somewhat roughly dentated border. When the deep colonies push forward to the surface of the gelatin they form white, elevated, drop-like masses, having a diameter of 1 to 2 mm. In gelatin stick cultures the gelatin is not liquefied.

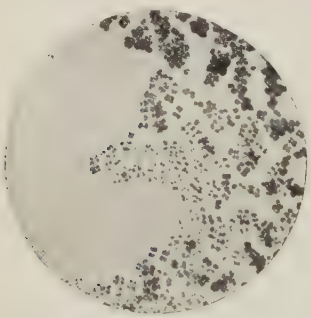


Fig. 96.—Micrococcus tetragenus. Stained with methylene blue. × 800 diameters.

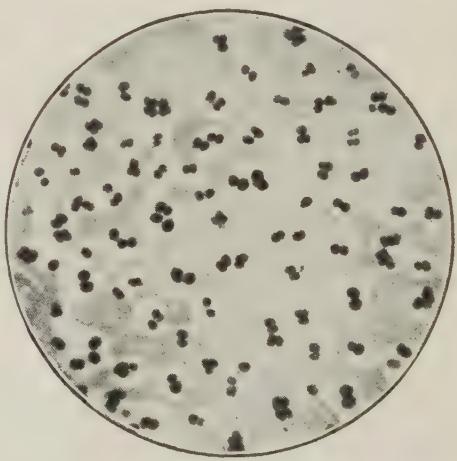


Fig. 97.—Micrococcus tetragenus from peritoneal fluid. Stained with fuchsin. (Fränkel.) × 1000 diameters.

Growth on Agar and Blood Serum.—The colonies appear as small translucent, round points, which have a grayish-yellow color and are slightly elevated above the surface of the medium.

Pathogenesis.—Subcutaneous injections of a culture of this micrococcus in minute quantity is usually fatal to white mice. The micrococci are found in comparatively small numbers in the blood of the vessels and heart, but are more numerous in the spleen, lungs, liver and kidneys. Intraperitoneal injections given to guinea-pigs and mice are followed by purulent peritonitis, beautifully formed cocci in groups of four being obtained in immense numbers from the exudate. Rabbits and dogs are not affected by large doses of a culture subcutaneously or intravenously administered. Spontaneous infection of guinea-pigs has shown a tetragenus which has been named Gaffkya tardissima.

In man it is generally non-pathogenic, except in the conditions already cited and then is probably a secondary invader. It has been reported in pure cultures from purulent inflammations a very few times. Byea and Nathan[1] found it in the pus of a case of osteoarthritis. Coyon and Lavedan[2] report obtaining it from a case of septicemia. It has been obtained by us as the only organism in a case of chronic conjunctivitis.

The serum from immunized cases has not been used therapeutically in human infection. Vaccines may be employed as with staphylococci.

THE STREPTOCOCCI.

All chain-forming, non-motile, non-spore-bearing, spheroidal bacteria that are insoluble in bile are placed in this group. The great majority of them are Gram-positive, also aërobic and facultative anaërobic. A few unnamed strains are Gram-negative and a few also little studied are anaërobic.

The pathogenic streptococci in their relation to human infection outweigh in importance most other disease-producing organisms. Besides many races of pathogens there are many saprophytic and parasitic varieties that must be placed in this group. Therefore from the practical standpoint of the clinician it is extremely important to be able as far as possible to separate the pathogenic forms from the non-pathogenic forms and to determine the relationship between the pathogenicity of the former and their other characteristics. Pure cultures of a streptococcus were obtained first by Fehleisen (1883) from a case of erysipelas. This chain coccus which he called *Streptococcus erysipelatis* was shown to be capable of producing erysipelas in man. Rosenbach (1884) and others isolated a streptococcus from the pus of acute abscesses and gave it the name of *Streptococcus pyogenes*. Then Klein (1886) and others isolated streptococci from scarlatina throats and called them *Streptococcus scarlatinæ*. Klein isolated his streptococcus from a case of scarlet fever occurring in an epidemic of septic sore throat which was traced

[1] Ann. de Méd., 1922, **2**, 193.
[2] Ibid., p. 209.

to milk. The first classification was thus based chiefly on pathogenicity. It was then thought that the streptococci of erysipelas, of acute abscesses, of septicemia, of puerperal fever, etc., belonged each to a different species, chiefly because they seemed to have the power each to produce a specific disease. But when it was shown that the same streptococcus might produce different lesions, *e. g.*, a strain from an abscess might also cause erysipelas, it was thought for a time that they might all be one species; and it is only recently that evidence is accumulating to show that these beginners may not have been far wrong in some of their specific names.

In 1903 Schottmüller made a broad grouping of the pathogenic forms on biochemical characteristics. His three types were: (1) *Streptococcus pyogenes* or *erysipelatis*, which shows hemolysis on blood agar plates; (2) *Streptococcus mitior* or *viridans*, which produces a green halo about the colonies on blood agar plates; and (3) *Streptococcus mucosus* which produces a mucoid growth and a dark green zone. Park and Williams (1905) and others since then, showed that Streptococcus mucosus should be placed with the pneumococci under the common name Pneumococcus mucosus. Gordon (1903) and Andrews and Horder (1906), using 9 test substances, chiefly carbohydrates, on over 1000 strains, divided streptococci from various sources into nine groups. Smith and Brown's[1] grouping (1915) and later Brown's,[2] based on erythrocyte reaction and morphology of colonies in a standardized erythrocyte plate is a very broad grouping. They attempted to show relationship between these characteristics and carbohydrate reactions as well as pathogenicity for rabbits. Holman's[3] classification is also based on erythrocyte and carbohydrate reactions and their correlation with pathogenicity. Avery and Cullen[4] state that the hydrogen-ion concentration at which human strains of hemolytic streptococci cease to grow (pH 5.2 to 5) is different from that of bovine strains (pH 4.5 to 4.3). More work has been done in the last few years on the relationship of specific serum reactions to other characteristics. Dochez, Avery and Lancefield[5] studied strains of hemolytic streptococci obtained from soldiers in a camp and found that they fall into six groups by the direct agglutination test. They

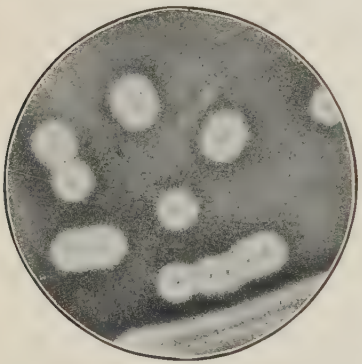


FIG. 98. — Twenty-four-hour growth of streptococci on surface of blood agar, showing zones of hemolysis around growth.

[1] Jour. Med. Res., 1915, **31**, 455.
[2] Monograph No. 9 of the Rockefeller Institute of Medical Research, 1919.
[3] Jour. Med. Res., 1916, **34**, 377 (with bibliography).
[4] Jour. Exp. Med., 1919, **29**, 215.
[5] Ibid., **30**, 179.

also stated that the serum of each type protected only its own type. The carbohydrate fermentations of the majority of all the strains were alike. Many investigations have been made on the relationships of agglutinins and the absorption of agglutinins to the different strains of this group. This question of agglutinating types will be taken up later.

A broad practical classification, such as that of Holman, Brown or Blake,[1] based on blood and sugar changes, is the following:

I. Produce definite hemolysis. (Streptococcus pyogenes of Rosenbach; St. hemolyticus of Rolly; "Type β (*beta*)" of Smith and Brown; St. hemolysans of Blake and others).

Sugars:

Lactose	Mannitol	Salicin.	
+	−	+	= St. pyogenes (Rosenbach, Gordon, Holman and others) divided into several ill-defined agglutinative groups obtained from most purulent processes.
+	−	−	= St. anginosus (Andrews and Horder) inflamed throats, scarlet fever and other lesions.
−	−	+	= St. equi (Schutz) from strangles in horse, occasionally from humans. Several other less frequent combinations of sugar fermentations (Andrews and Horder, Holman and others).
−	−	−	= St. subacidus from occasional erysipelas.

II. Produce no definite hemolysis.

A. Produce methemoglobin. Streptococcus viridans "Type α (*alpha*)" (Smith and Brown's).

Sugars:

Lactose	Mannitol	Salicin.	
+	−	+	= St. mitior (Schottmüller) St. mitis (Andrews and Horder, Holman).
+	+	+	= St. fecalis (Andrews and Horder, Holman and others) St. Lactis.
+	−	−	= St. salivarius (Andrews and Horder).
−	−	+	= St. equinus (Andrews and Horder).

B. Produce no methemoglobin under comparative conditions. Stianhemolyticus (Zangmeister). St. saprophyticus (Mandelbaum) and "Type γ (*gamma*)" (Brown).

Sugars: Too few strains have been studied with sugar to make a general grouping. (See Brown's Table II.[2])

A number of intermediate types have been described.

[1] Jour. Med. Res., 1917, **36**, 99.

[2] Brown, J. H.: Monograph No. 9 of the Rockefeller Institute of Medical Research, 1919.

Hemolytic Streptococci.—Hemolytic streptococci from various sources all present somewhat similar morphological and cultural appearances. Minor differences may distinguish certain serological groups or certain pathological conditions, but they may not be constant. Indeed, all streptococci show a great tendency to dissociate or break up into coarse and fine (rough and smooth) colonies, some of which may show other characteristics different from the parent colony.

An attempt has been made by Bergey and others to distinguish species in this group by the absorption of agglutinins, and connoting a single species with definite disease complex. Our work has shown, however, that there are too many agglutinative types among the strains of hemolytic streptococci obtained each from one of a series of the same named disease and too many different named diseases caused by the same agglutinative type to consider a single agglutinative type the cause of a single named disease. Thus we have found that the majority of strains from scarlet fever fall into four agglutinative types leaving a number of strains untyped, that strains from erysipelas fall into two agglutinative types with a small percentage of strains left over, that strains from our Type IV group scarlet fever cross absorb with strains from our Type I group erysipelas, that some strains from puerperal fever and from streptococcus meningitis cross absorb with some of our scarlet fever type strains respectively.

Furthermore we have found that strains of hemolytic streptococci from an outbreak of septic sore throat and from the cow to which the epidemic was traced cross absorb with our largest scarlet fever group.

Hemolytic strains in general may be described as follows:

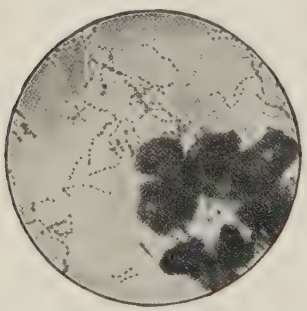


FIG. 99.—Streptococci in peritoneal fluid, partly enclosed in leukocytes. × 1000 diameters.

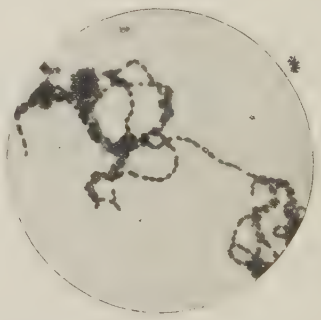


FIG. 100.—Streptococcus growing in long chains in bouillon culture. × 1000 diameters.

Morphology.—The cocci, when fully developed, are spherical or oval. They have no flagella or spores. They vary from 0.4μ to 1μ in diameter. They vary in dimensions in different cultures and even in different parts of a single colony. They multiply by binary division in one direction only, forming chains of eight, ten, twenty, and more elements, being, however, often associated distinctly in pairs. On solid media the cocci

occur frequently as diplococci, but usually they grow in longer or shorter chains. Frequently certain cocci exceed their fellows greatly in size, especially in old cultures. Hueppe called these arthrospores. Some varieties have a capsule-like appearance when growing in the blood and in blood-serum media.

Staining.—They stain readily by anilin colors. They give a positive reaction by Gram's method, but are not usually as Gram-positive as are viridans strains.

Physiology.—Streptococci of the hemolytic type grow comparatively readily in various liquid and solid culture media. The most favorable temperature for their development is from 30° to 37° C., but they multiply to some extent at ordinary room temperature—18° to 20° C. They are facultative anaërobes, growing both in the presence and absence of free oxygen.

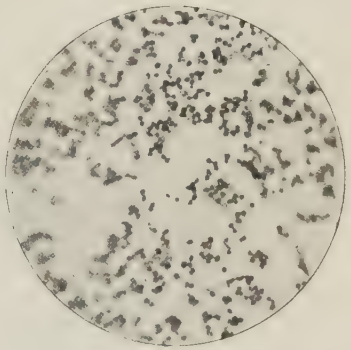


FIG. 101.—Streptococci from solidified serum culture appearing mostly as diplococci. × 1000 diameters.

Growth on Gelatin.—Tubes of gelatin which have been inoculated with certain strains by puncture with a platinum needle show on the surface, little growth beyond the point of entrance. In the depth of the gelatin on the second or third day a distinct, tiny band appears with granular edges or made up of granules. These granules may be very fine or fairly coarse. They are nearly translucent, with a whitish, yellowish, or brownish tinge. The gelatin is not liquefied.

Growth on Agar.—On agar plates the colonies are visible after twelve to thirty hours' growth at 37° C., and present a beautiful appearance when magnified sufficiently to see the individual cocci in the chain, looped or not looped, at the margin of the colonies. The colonies are small, not averaging over 0.5 mm. in diameter (pin-head). From different sources they vary in size, thickness, mottling, color, and in the appearance of their borders. The streptococcus growing in short chains shows but little tendency to form true loops, but rather projecting rows at the edges of the colonies.

Growth in Blood Media.—For diagnostic as well as other purposes, whole blood should be added to media for growing streptococci. Blood agar on which the cocci can be streaked or into which they can be mixed in appropriate dilutions gives the most differential picture. (See under Media.)

Growth in Bouillon.—Most streptococci of this type grow well in slightly alkaline bouillon at 37° C., reaching their full development within thirty-six to forty-eight hours. Those which grow in long chains usually give an abundant flocculent deposit and leave the liquid clear. The

deposit may be in grains, in tiny flocculi, in larger flakes, or in tough, almost membranous masses, the differences depending on the strength of union between the pairs of cocci in the chains. Some of the streptococci growing in long chains, however, cause the broth to become cloudy. This cloudiness may be only temporary or it may be lasting. Those growing in short chains, as a rule, cloud the broth, this cloudiness remaining for days or weeks. A granular deposit appears at the bottom of the tube. An addition of 0.5 to 1 per cent. glucose aids the development of streptococci, but the acid produced tends later to hasten their death and to make them lose virulence. A trace of calcium aids the growth as it neutralizes some of the acids produced. This is best added as a piece of marble. In order to overcome the spontaneous agglutination that interferes with the agglutination test we use a balanced phosphate broth (Dochez, Avery and Lancefield[1]) *to which we add* 0.1 *per cent.*

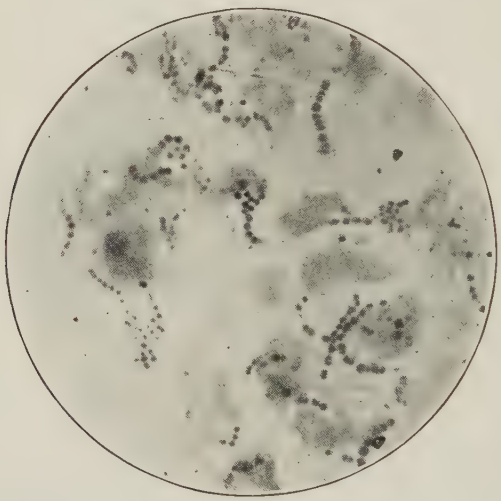


Fig. 102.—Streptococci in cerebrospinal fluid from a case of meningitis. (Neal.) × 1500.

dextrose, transplant every day until we get a homogenous cloud. So far our most refractory strains have responded to this treatment.

Growth in Ascitic or Serum Bouillon.—The development in this, which is one of the best mediums for the growth of all streptococci, is more abundant than in plain bouillon. The liquid is usually clouded, and a precipitate occurs after some days, the fluid gradually clearing. The addition of blood serum frequently causes streptococci, growing in short chains in nutrient bouillon, to produce long chains. The reverse is also true, and in the blood all forms are usually found, some, at least, being diplococci or in short chains. A culture which will not grow well in either ascitic fluid or serum bouillon may grow well in broth containing both.

[1] Jour. Exp. Med., 1919, **30**, 171.

Growth in Beef Heart Bouillon.—Bouillon to which about one-third beef heart has been added makes a fine medium for streptococci.

Duration of Life Outside of the Body.—When dried in blood or pus they may live for several months at room temperature. When precipitated from blood or pus or fluid media they are frozen and dried in a vacuum; the streptococci remain alive for years and retain their characteristics while frozen and kept dry. In gelatin and agar cultures they live for from one week to three months. In order to keep such cultures alive and vigorous, it is best to transplant them frequently. They may be kept alive for a long time in semisolid agar stick cultures at room temperature or in serum or ascitic fluid bouillon in small sealed glass tubes in the ice-chest.

Resistance to Heat and Chemicals is given under Disinfection, Part III.

Production of Toxic Substances.—There is no doubt that certain hemolytic streptococci cause fever, general symptoms of intoxication and death by means of toxic substances.

Four different kinds of poisons have been described.

1. *Streptohemolysin.*—Ever since Marmorck (1895) observed the hemolyzing of the red blood cells by certain streptococci this phenomenon has been the subject of study. We may summarize our present ideas of it as follows:

(*a*) The substance causing hemolysis is produced early in the growth of the organism, in eight to eighteen hours. It is not very stable and when produced in fluid culture media, *e. g.*, in ascitic broth, it should be filtered off after about eighteen hours growth. It may have disappeared about fourteen hours later. The production curve varies with the age and amount of culture inoculated and with the kind of medium used (Besredka,[1] Rudiger,[2] McLeod,[3] Lyall[4] and others). Some of it may be lost by absorption during the filtration. This loss may be avoided by using the high power centrifuge to separate the organisms (Nakayama,[5] Dekruif and Ireland[6]) from the culture fluid.

(*b*) No specific antibody has been obtained for it (Besredka,[1] Rudiger,[2] and others). In these characteristics it differs from staphylolysin.

(*c*) Some normal serums have the property of neutralizing its action but the nature of this property is not known.

It may be demonstrated within blood agar plates or in fluid containing blood in test-tubes. The plate method is as follows: If 1 c.c. of fresh or defibrinated blood is added to 6 c.c. of melted agar at 40° to 45° C., well shaken, inoculated with characteristic streptococci and poured into a Petri dish there will appear in twelve to twenty-four hours tiny colonies surrounded by clear zones of about $\frac{1}{4}$ to $\frac{1}{3}$ inch in diameter proceeding immediately from the margin of the colony.

The tube method is used for quantitative determination of hemolysis. The titration is made by adding decreasing amounts of a definite

[1] Ann. de l'Inst. Past., 1901, **15**, 880.
[2] Jour. Am. Med. Assn., 1903, **41**, 962; Jour. Inf. Dis., 1906, **3**, 663 and 755.
[3] Jour. Path. and Bact., 1912, **16**, 321.
[4] Jour. Med. Res., 1914, **30**, 515.
[5] Jour. Inf. Dis., 1919, **25**, 509.
[6] Jour. Inf. Dis., 1920, **26**, 285.

culture (eighteen-hour 2 per cent. peptone ascitic broth culture, according to Lyall), to a constant quantity of washed red blood cells (1 c.c. of a 5 per cent. suspension of sheep's red cells: Lyall). The tubes are incubated in water-bath at 37° C. for one hour and readings are then made.

Pneumococci and many streptococci grouped as green *streptococci*, which occur together with hemolytic forms in the throat, lungs, and elsewhere, on the other hand, produce only narrow zones of a green pigment. Anthony[1] and others have found that from a streptococcus producing abundant hemolytic substances strains may be obtained, by selecting certain colonies, which fail to make them. She has not been able to obtain from strains producing in first cultures the green pigment only any strains producing hemolytic substances. Rosenow, on the other hand, claims that he can readily change certain non-hemolytic strains into hemolytic strains.

Valentine[2] found that certain colonies of hemolytic streptococci in blood plates showed only a green zone. The cultures from these seem to have lost the power to produce hemolysis, *but they were still agglutinatively like the hemolyzing colonies.*

2. *Streptoleukocydin.*—Soon after staphyloleukocidin was demonstrated, Rudiger,[3] showed that streptococci also produced a poison for leukocytes. A few others have studied it since then. The following is a summary of this work.

(*a*) The leukocidin follows the same curve as the hemolysin in the time of its appearance in cultures (Rudiger,[3] Hektoen,[4] Nakayama[5]).

(*b*) Its action on leukocytes is tested in the same way Neisser and Wechsberg, (1901), tested for staphyloleukocidin, that is, the reducing power of live leukocytes for methylene blue was made use of (Rudiger, Hektoen, Nakayama). Nakayama describes the process of obtaining fresh active leukocytes (see p. 275) as follows: The solution of methylene blue is made by dissolving 1 c.c. of the strain in 29 c.c. of distilled water and 20 c.c. of absolute alcohol.

Different dilutions of the leukocyte suspension are made in normal salt solution (0.9 per cent.). Two drops of methylene-blue solution are added to each tube and the mixture is covered with liquid paraffin and put in incubator at 37° C. for two hours. If reduction occurs the solution becomes colorless, if none occurs the color remains green. Properly graded dilutions will show the minimum number of leukocytes that cause a reduction of the methylene-blue. To show the presence of leukocidin, different quantities of the fluid to be tested are added to twice the numerical quantity of leukocytes that caused reduction and these mixtures are placed in the incubator at 37° C. for one and a half hours. Then 2 drops of the methylene-blue solution are added to each tube, the mixtures are covered with liquid paraffin and returned to the incubator for two hours when readings are made. The presence of a

[1] Jour. Infect. Dis., 1909, **6**, 332.
[2] Jour. Exp. Med., 1922, **36**, 157.
[3] Jour. Am. Med. Assn., 1905, **44**, 198.
[4] Ibid., 1906, **46**, 1407.
[5] Jour. Inf. Dis., 1920, **27**, 86.

green color indicates that the leukocytes have been hindered from reducing the methylene blue.

(*c*) Destroyed by heat at about 60° C. for thirty minutes and cannot be reactivated, in this like known exotoxin (Nakayama[1]). Streptolysin retains its power when so heated.

(*d*) Power to produce leukocidin is paralleled by the virulence—while that to produce streptohemolysin has no relation to virulence. Neither do all cultures producing hemolysin produce leukocidin (Nakayama[1]).

(*e*) When a mixture of leukocytes and leukocidin is kept at low temperature (ice-box about 4° C.) they combine leaving a filtrate which is no longer leukocyte destroying, but may be hemolytic.

(*f*) Normal serums of animals (human and horses to higher degree) are able to neutralize the action of leukocidin to some degree. Anti-streptococcus serum has no greater action but a serum produced by the inoculation of leukocidin seems to have an increased power to neutralize. It seems also to promote phagocytosis.

(*g*) Streptoleukocidin does not neutralize the effect of staphylo-leukocidin (Nakayama[1]).

3. *Filtrable Toxic Products and Exotoxins.*—Almost as far back as the discovery of streptococci have investigators claimed to have obtained toxic filtrates from cultures of streptococci. The work of Savchenko (1905) and others will be given below. Clark and Felton,[2] in 1918, described "a filtrable toxic product of the hemolytic streptococcus" produced in growths in rabbits' whole blood diluted with Locke's solution. This in doses of 0.1 to 1 c.c. produced death in rabbits.

In 1924 the Dicks,[3] showed that filtrable toxin formed in fluid cultures of scarlatina strains of hemolytic streptococci produces a specific skin reaction in humans and is neutralized by scarlatina convalescent serum and by specific antitoxic serum. (See below.)

Their work was soon corroborated in the main throughout the world. But the specificity of the reaction was quickly shown by Kirkbride and Wheeler,[4] Park and Spiegel[5] and others to be complicated by the fact that the toxin gives evidence of being complex—made up of different components shown by its inability to be neutralized in all people by a monovalent antitoxin.

Birkhauk and others claim that a specific serum may be stimulated by the injections of strains of hemolytic streptococci from erysipelas. Our work as well as that of others seems to show that the exotoxins produced have a *much broader* quality than have the other antigens. In other words, an antitoxic serum made from any one strain may have equal neutralizing effect on many strains from different sources and belonging to different agglutination types.

[1] Jour. Inf. Dis., 1920, **27**, 86.
[2] Jour. Am. Med. Assn., 1918, **71**, 1048.
[3] Ibid., 1924, **82**, 265.
[4] Proc. Soc. Exp. Biol. and Med., 1924, **22**, 85.
[5] Jour. Immunol., 1925, vol. **10**, No. 5.

For example, we injected 63 children each with a skin-test dose[1] of toxin from our Type I scarlet fever, Type I erysipelas and Type II erysipelas respectively, with the following results:

Number of Cases	Type I scarlet fever	Type I erysipelas	Type II erysipelas
22	−	−	−
12	+	+	+
6	+	+	−
12	+	−	−
3	+	−	+
4	−	−	+
4	−	+	−

33 or 52 per cent = + with Type I scarlet fever toxin.
22 or 35 per cent = + with Type I erysipelas toxin.
19 or 30 per cent = + with Type II erysipelas toxin.

These toxins were tested for neutralization on 3 children. The first 2 were neutralized in all 3 children; the third was only neutralized in 1 child.

Ferry showed that a viridans type of streptococcus also made a toxin which produced and was neutralized by an antitoxin. This indicates that many types of streptococci make more or less extracellular toxins.

4. *Poisons in the Cell Substance of Streptococci (Endotoxins).*—The bodies themselves of streptococci seem to be only slightly toxic.

Other Antigens of Streptococci.—They form agglutinogens and precipitogens. They also form an opsonin-producing substance and a substance lytic for themselves (bacteriophage).

The Production of Agglutinins.—Increasing inoculations of living or dead streptococci into rabbits or other suitable animals will usually produce a considerable amount of agglutinin. Until recently, however, every effort to overcome the interfering factor of self-agglutination, shown by as many strains of streptococci, has met with only partial success. Every one of the many strains we have studied in the last few years has formed a satisfactory even suspension of cocci by the following method. The stock cultures are kept on chocolate agar (see Media), transplanted about once a month. When ready to start an antigen the culture is transplanted every day into small tubes of phosphate broth plus 0.1 per cent. dextrose in solution (sterilized in the autoclave) until the growth becomes uniformly cloudy. Fresh potato broth[2] may give an even suspension in a shorter time. The time needed for this varies from a few days to about a month. So far, every strain we have, however, self-agglutinating originally by these methods finally forms a uniform suspension. Recently isolated strains are less self-agglutinative.

Tunnicliff followed by Bliss[3] and then Gordon[4] later reported that

[1] A skin-test dose, the amount of toxin that will produce a skin reaction of 1.5 cm. diameter in the average of susceptible children.

[2] Dochez and coworkers: Jour. Exp. Med., 1919, **29**, 215.

[3] Johns Hopkins Hosp. Bull., 1920.

[4] Brit. Med. Jour., 1921.

of the strains of hemolytic streptococci isolated from scarlatina, 80 per cent. fell into one agglutinative type. But only Gordon among these reported complete absorption (not cross absorption) tests with those strains and he worked with only 9 case strains. We have isolated hemolytic streptococci from over 100 cases of scarlet fever, early in the disease, and by complete cross absorptions have found only 35 per cent. of the strains falling into one group. Among the other strains we found three more groups. Griffith and others agree that there are at least four serological types among scarlet fever strains. We have also found that 15 per cent. of strains from non-scarlet fever cases fall into our largest scarlet fever group.

FIG. 103.—"Coarse" and "fine" colony of growths of hemolytic streptococci fished from one strain.

The question of the relationship of these types to those from other diseases is still under investigation.

We have found that the majority of strains from erysipelas also fall into two groups by the absorption of agglutinins, one larger one and one smaller one, leaving several strains still ungrouped.

We have found further that our group Type IV scarlet fever strains cross absorb with our group Type I erysipelas strains.

We have also found that certain strains from puerperal fever cross absorb with our Type I erysipelas and other strains fall into our largest scarlet fever group. The same grouping occurs with strains for meningitis.

We have found evidences of sub-relationships which give us hope

that we may obtain a few master strains. We have found that our Type I scarlet fever and our Type I erysipelas (Birkhauk's C 3) are master strains for a number of strains from different sources.

The most important question of the relationship of these strains to specific exotoxin production will be considered below. This is rendered more difficult from the fact that a streptococcus produces not a single exotoxin, but a number of them.

The whole question of the relationship of these strains from the standpoint of agglutination is further complicated by the fact that each strain has a tendency to break up into different varieties. One indication of this, now known to be a universal trait of microbes, is the production of "coarse" and "fine" or "rough" and "smooth" colonies.

Complement Fixation.—The method and results of this test have been considered in Chapter XI.

Pathogenesis.—The majority of test animals are not markedly susceptible to infection by hemolytic streptococci directly from human beings. White mice and rabbits are the most susceptible, and these animals are therefore usually employed for experimentation. These streptococci, however, differ greatly in the effects which they produce in inoculated animals, according to their animal virulence. The most virulent when injected in the minutest quantity into the circulation or into the subcutaneous tissue of a mouse or rabbit, produce death by septicemia. Those of somewhat less virulence produce the same result when injected in considerable quantities. Those still less pathogenic produce usually a mild septicemia when injected into the circulation, and when injected subcutaneously, they produce abscess or erysipelas (see below for Gay's work). The remaining streptococci, unless introduced in quantities of 20 c.c. or over, produce only a slight redness, or no reaction at all, when injected subcutaneously, and little or no effect when injected directly into the circulation.

One important fact that experience teaches us is that those streptococci which are the most dangerous are those which have come immediately from septic conditions, and the more virulent the case the more virulent the streptococci are apt to be for animals of the same species. There seems also to be a strong tendency for a streptococcus to produce the same kind of an inflammation, when inoculated, as the one from which it was obtained; for example, streptococci from erysipelas tend to produce erysipelas, from septicemia to produce septicemia, etc. This question of the localization of streptococci in the special tissue from which they were obtained, that is, their elective localization, has been much studied, especially by Rosenow. Gay has made some very suggestive studies on this subject. The relationship of this apparent power to true mutations is still undecided.

Occurrence Spontaneously in Animals.—Besides streptococci similar to those in man, animals are infected by strains that are negative to Gram and fluidify gelatin. Udder infections of the cow and glandular diseases of the horse are frequently due to these. The streptococcic

inflammations in animals are almost as frequent and serious as they are in man. Epidemics have been reported in test animals. This must be remembered in any experimental work on streptococci. During the last year we have had several outbreaks of infection among our white mice due to hemolytic streptococci.

Occurrence in Man.—Hemolytic streptococci have been found to be a primary cause of infection in the following diseases: Erysipelas and scarlet fever and certain cases of circumscribed and extensive acute abscesses, impetigo, cellulitis (circumscribed as well as diffused), sepsis, puerperal infection, acute peritonitis, angina and septic sore throat, bronchopneumonia, periostitis, osteomyelitis, synovitis, otitis media, mastoiditis, enteritis, arthritis, meningitis, pleurisy, empyema and endocarditis. They have also frequently been found as the secondary infection in many diseases, such as pulmonary tuberculosis, bronchopneumonia, septic diphtheria, measles, smallpox and others.

In diphtheritic false membranes this type of micrococcus is very commonly present, and is frequently the source of deeper infection, such as abscesses, pneumonia and septicemia, and in certain cases accompanied by a diphtheritic exudation, in which the Löffler bacillus has not been found by competent bacteriologists, it seems probable that the *Streptococcus pyogenes,* alone or with other pyogenic cocci, is responsible for the local inflammation and its results. These forms of so-called diphtheria, as first pointed out by Prudden, are most commonly associated with scarlatina, measles, erysipelas and phlegmonous inflammation, or occur in individuals exposed to these or other infectious diseases. Varieties are regularly found in the throat secretion of healthy individuals. Pilot and Davis,[1] among others, have found that the tonsillar crypts in apparently normal cases contain hemolytic streptococci in almost 100 per cent. of the many examined, while the surface tonsils may show few.

Septic Sore Throat.—Streptococcus infection of the throat appears at times as a severe epidemic. Most of these epidemics can be traced through the milk supply to a human carrier, as Smith, Brown,[2] Krumwiede and Valentine[3] and others have shown. In some of the outbreaks cases develop rashes like those of scarlet fever. Streptococci of human origin may invade the milk ducts and multiply in the udder without causing physical signs of mastitis. The bovine streptococci commonly producing mastitis (Mathers[4]) have probably no relation to septic sore throat. During 1928 we had the opportunity of comparing the strains of hemolytic streptococci from two severe epidemics of septic sore throat and one of scarlet fever with our type strains from scarlet fever. In the one instance we found that by the absorption of agglutinins the strain from the cow and from the human carrier each absorbed the agglutinins from our Type Sub. I scarlet fever serum. Strains from the

[1] Jour. Inf. Dis., 1919, **24**, 386.
[2] Jour. Med. Res., 1915, **31**, 455.
[3] Ibid., 1915, **33**, 231.
[4] Jour. Infect. Dis., 1916, **19**, 222

cow and the human from the other epidemic of septic sore throat and from the one of scarlet fever gave each a toxin that was neutralized by our scarlet fever antitoxin. Jones and Little[1] obtained from the udder a streptococcus of a scarlet fever type. We have not tested strains of the so-called Streptococcus epidemicus of Davis[2] serologically.

Brown, Frost and Shaw[3] have recently studied the types of hemolytic streptococci in milk.

Scarlet Fever.—The etiology of scarlet fever has been a subject for discussion for many years. Soon after the discovery of the relation of the diphtheria bacillus to diphtheria Löffler[4] noted that long-chained streptococci were very abundant in the throats of scarlet fever patients. The fact that a scarlet fever-like rash developed in certain cases of wound infection and puerperal fever, which are recognized as being due to streptococci, strengthened the opinion that there was a close relationship between certain of these streptococci and scarlet fever. Indeed, Klein,[5] in 1885, advocated naming what he called a special long-chained streptococcus, the Streptococcus scarlatinæ. Repeated attempts by numerous investigators to produce scarlet fever in animals, by the inoculation of throat secretion or virulent cultures of hemolytic streptococci obtained from the throats of scarlet fever patients, failed.

Moser, in 1902, published a report of the good therapeutic results obtained by the use of antiscarlet fever serum produced by the horse after it had received repeated injections of living streptococci obtained from scarlet fever cases together with the broth in which they had developed, so as to get the antigenic value of the culture as well as any possible toxins that might have developed in the broth. Schick,[6] three years later, after an extensive experience, makes a report on the use of the Moser serum. He states: "The surprisingly good effect on the cerebral and the general symptoms, the disappearance of the cyanosis, the rapid decline of the intensity of the rash and of the temperature and the disappearance of the diarrhea can be considered as due only to antitoxic effects."

A Russian, Savchenko, in 1905, proved that the filtered broth in which the culture had grown actually contained a strong toxin, which could be neutralized by the serum of an immunized horse.

We now turn to the problems of immunization against scarlet fever and the value of the evidence so obtained in favor of the causal relationship of the streptococci to scarlet fever. Gabritschewsky[7] finished an experimental study on a streptococcic infection of horses and believed he had proved that a vaccine of toxin and dead streptococci was able to develop a moderate degree of immunity. He then turned to the

[1] Jour. Exp. Med., 1928, **47**, 945. [2] Jour. Am. Med. Assn., 1912, **58**, 1853.

[3] Brown, Frost and Shaw: Jour. Inf. Dis., 1926, **38**, 381. Frost, Brown and Thomas: Jour. Inf. Dis., 1927, **40**, 698.

[4] Arb. a. d. k. Gsndhtsamte, 1884. [5] Proc. Roy. Soc., 1887.

[6] Ueber die weiter Erfolge der Serum, behandl. Scharlach, Deutsch. med. Wchnschr., December 28, 1905.

[7] Centralbl. f. Bakteriol., , L. orig., vol. **41**.

problem of immunization against scarlet fever, utilizing for injections a streptococcus obtained from scarlet fever. His vaccine was made of bouillon, in which the streptococci had grown for four days, plus 3 per cent. by volume of the streptococci. The toxic bouillon with the streptococci was heated to 60° C. to kill the streptococci and was then preserved with 0.5 per cent. carbolic acid. Three doses were given subcutaneously one week apart. For infants under two years the dose was 0.25 c.c.; for those between two and ten years the dose was 0.5 c.c., and for those over ten years, 1 c.c. Three injections were given *one* week apart and the doses were doubled each time. The results in children were most interesting. Langowoi found that of 120 children inoculated, in 13.3 per cent. there developed, after the first dose, a fine scarlet fever-like exanthema which was not followed by desquamation. A few developed a sore throat and strawberry tongue and fewer still, vomiting. He states these symptoms which are characteristic of scarlet fever add an extremely important proof for the specificity of the scarlet fever streptococcus and its toxin. He found that the second and the third injection, although they were twice and four times as large as the first, rarely produced a rash. This he considered as evidence of the rapid development of immunity.

Gabritschewsky,[1] in 1907, gives further evidence in favor of vaccination from the work of his followers. He made a very important finding, in that 26 children who had previously had scarlet fever showed either no reaction or but a slight local swelling.

Gabritschewsky considered that the results of his investigations, together with that of other workers, proved that the scarlet fever streptococcus produced its toxins in the throat. The absorbed toxins produced the rash, the fever and the other symptoms. The lowered resistance allowed the scarlet fever streptococci or other streptococci to invade distant parts and cause the so-called complications. An additional evidence[2] of the casual relationship was the development of scarlet fever by the accidental inoculation, in our laboratory in 1912, of the throat of a laboratory assistant with a mixed culture of several strains of streptococci, including two hemolytic strains from scarlet fever. Two days later a pharyngitis developed and on the following day a typical scarlatinal rash. It ran the characteristic course of a moderate case of scarlet fever.

Then followed the discovery of Schultz[3] and Charlton, who, in 1918, described the reaction known by their name. They found that when 1 c.c. of a slightly diluted convalescent serum is injected intracutaneously into the reddened skin of a scarlet fever patient a local blanching occurs.

This brings us to the discoveries of the Dicks and Dochez. In October, 1923, Dick and Dick[4] report the results of inoculating 5 volunteers, who had lived in the country, with a streptococcus culture

[1] Berl. klin. Wchnschr., 1907, **44**, 85, 556.
[2] Arch. Int. Med., 1914, **13**, 909.
[3] Ztschr. f. Kinderh., 1918, **16**, 328.
[4] Jour. Am. Med. Assn., 1923, **81**, 1166.

from a case of scarlet fever. One developed a typical case of moderate scarlet fever. Five others were inoculated with the filtrate of a broth culture. None of these developed the disease, but when these same individuals were inoculated with the streptococcus one developed it. This result indicated that there was no living filtrable virus in the culture in addition to the streptococci. Simultaneously came the work of Dochez described below. The Dicks developed the Dick test by using the toxic filtrate of a broth culture of the scarlet fever streptococci intracutaneously for the determination of immunity, just as the Schick test is used to determine immunity in diphtheria. Their skin test provides also a means for testing the antitoxic potency of the serum. These experiments of the Dicks made clearer the necessary proof to establish that scarlet fever is caused by a group of special hemolytic streptococci. There can be no question that, while Moser, Gabritschewsky and Savchenko discovered the casual relation of the scarlet fever streptococcus to scarlet fever and made an antitoxic serum and a vaccine, the world did not properly appraise their findings and that to the Dicks and Dochez belongs the credit of adding convincing evidence of the relationship of the streptococcus to scarlet fever.

The Dick Test.—Dick and Dick found that weak solutions of the toxin may be employed in skin tests to determine susceptibility to scarlet fever. The toxin is first carefully standardized, and diluted so that 0.1 c.c. represents a skin-test dose. The test consists of an intradermal injection of exactly 0.1 c.c. of the skin-test dilution on the flexor surface of the forearm. The reaction is observed at the end of twenty-four hours. An area of reddening, 1 to 2 cm. in diameter, after injection of the standard dose represents from some degree of susceptibility to complete susceptibility to scarlet fever. The disappearance of a reaction within twenty-four hours or an area of redness less than 1 cm. in diameter indicates probable immunity.

Dick and Dick[1] found that, by proper dosage, they were able to immunize persons with positive skin tests so that their skin tests became negative, and they did not contract scarlet fever on exposure.

They report a series of 125 persons exposed to scarlet fever. In this series 63 who showed negative skin reactions were not immunized. Fifty-two had positive skin tests, and were immunized with toxin. None of these persons contracted scarlet fever. They emphasize the importance of carrying the immunization to the point of a negative skin test. By employment of five doses of toxin they have been able so to divide the toxin as to eliminate severe reactions.

The results of the Dick test have been remarkably reliable. The Dicks[2] have reported that they have observed 158 nurses who gave a negative Dick test and who were later exposed to scarlet fever for periods of six weeks. None of these developed scarlet fever. Also no cases occurred among 230 persons with negative tests, although they had direct and prolonged exposure. During this time 9 persons

[1] The Prevention of Scarlet Fever, Jour. Am. Med. Assn., 1924, **83**, 84.
[2] Jour. Am. Med. Assn., 1925, **84**, 1477.

who had positive reactions developed scarlet fever. Our own studies with the Dick test have agreed with those of the Dicks in all essential points.

The Pseudo-reaction.—The scarlet fever toxin is very resistant to heat so that even two hours' boiling will not completely destroy it. This was not appreciated at first and we know now that a certain number of reactions which were believed by us to be pseudo-reactions were really due to the reaction in extremely susceptible persons of considerable traces of toxin left after boiling for one and a half hours. That it was the remains of the toxin and not the minute amounts of horse serum, as suggested by the Dicks, is proven by the fact that most of our tests were done with toxin which contained no horse serum and by the complete lack of reaction of the same dose of toxin when given with an adquate amount of convalescent human serum. Besides these false pseudo-reactions there are the real pseudo-reactions. We have found them in about 5 per cent of the cases. They are generally very moderate in amount and usually less than 1 cm. in diameter, so that the majority would not be considered positive cases, even if no control test was made. They occur more often in those who have received immunizing injections.

The routine control test for the pseudo-reaction is made with the same amount of toxin, heated to the boiling-point for two hours, which was used for the Dick test. For careful research work it is better to use also a boiled toxin to which has been added 5 per cent. of a potent convalescent scarlet fever serum. The control test in my opinion is not necessary for ordinary testing in children.

Evidence from the Blanching Test.—While the Dicks were making more definite the proof as to the causal relationship of scarlet fever streptococci to scarlet fever, Dochez was doing the same in developing a strong scarlet fever antitoxin, through a method, developed by himself, of injecting into horses subcutaneously and repeatedly small amounts of melted nutrient agar infected with scarlet fever streptococci. The serum of horses which had been subjected to such injections were found to give the Schultz-Charlton test in the skin of a patient, even when the serum had been diluted one thousand times. The horses were found to vary in their antitoxic potency. The serum was used by Blake[1] in cases in New Haven and by us in New York City. The same results were obtained as had been obtained twenty years ago by Moser and Savchenko, but now the potency of the serum could be estimated and the striking results with the Schultz-Charlton rash blanching reaction were a demonstration to all that antitoxin was truly present. The interest developed by Dochez's[2] announcement caused a number of biological plants to use his methods, while others utilized toxin standardized by the Dick test or crude toxic broth and dead organisms after the manner of Moser and Savchenko.

The Dick method develops only an antitoxic serum, while the others develop also antibodies against the bacteria.

[1] Jour. Am. Med. Assn., 1924, **82**, 712.
[2] Proc. Soc. Exp. Biol. and Med., 1924, **4**, 184.

In our opinion the antitoxic properties are of the greater importance in cases treated early, while the antibacterial properties are the more important in cases in which the toxic stage has changed to the septic stage. We use the Dochez method for the immunization of the horses, but in addition give occasional doses of toxin subcutaneously for the purpose of increasing the potency of the antitoxin. On page 189 a unit is defined as the amount of antitoxin which is required to neutralize 50 skin-test doses of scarlet fever toxin when they are mixed together, allowed to stand a few minutes and then injected intracutaneously. To test for the antitoxic potency of an unknown serum we add to a number of vials 100 skin-test doses of scarlet fever toxin contained in 1 c.c. of salt solution. To each vial we add 1 c.c. of salt solution containing a definite amount of the antitoxic serum to be tested. One-tenth of 1 c.c. of this mixture is injected intracutaneously in a child. At the same time a Dick test is made to be certain whether the case is susceptible and the same amount of the heated toxin is used to test the child's reaction to protein and finally the quantity of serum used in the potency test is also injected to test whether the skin is sensitive to the serum. If the patient gives a positive Dick test, and no protein or serum reaction, the test is reliable. The mixture containing the least amount of antitoxic serum which shows no skin reaction indicates the potency. Dividing the number of skin-test doses neutralized by 1 c.c. of serum by 50, gives the number of units in 1 c.c. The final result of the tests gives us an approximately accurate estimate of the amount of antitoxin. The antibacterial tests are done in a way similar to that used in testing anti-pneumococcic serum.

The Therapeutic Use of the Serum.—In moderate cases 5000 to 10,000 units of antitoxin are given intramuscularly. In severe toxic cases 10,000 to 20,000 are given intravenously. Unlike the practice in diphtheria, additional doses are given if the temperature does not fall within twelve hours to nearly normal or if it rises again. One reason for this is that the antitoxin solution is not as potent as is diphtheria antitoxin. A unit of scarlet fever antitoxin has much less neutralizing value than a unit of diphtheria antitoxin. Further, the individual human cases differ greatly in the amount of toxin present in the body; if as great a dose were given to every case as would suffice for the most severe one, an excessive dose would be given to the majority. There seems to be no danger in this delay, as the scarlet fever toxin does not combine with the tissues as firmly as does the diphtheria toxin. The therapeutic results have been excellent in most cases. There is no question as to the value of this serum. We use it in all cases that show marked toxic symptoms or that have a rectal temperature of over 102° F.

The Preventive Use of Serum and Vaccine.—We seldom use antitoxin as a preventive (passive immunization) in scarlet fever contacts. This is partly because the response to the vaccine by active immunization is so quick, much quicker than that following diphtheria vaccine, and then the disease itself is at the present time in this country mild. Those who receive an immunizing dose of serum are more liable to serum sickness than those receiving diphtheria antitoxin.

Active Immunization.—Earlier in this section we gave the work of Gabritschewsky and his followers. The Dicks have carried on a series of studies on the amount of toxin necessary to produce a durable immunity. At first smaller amounts were used than those advised by Gabritschewsky. It was found that while a small amount of toxin will produce immunity, it will not be lasting. Thus, in an institution we got the results given in the table:

RESULTS OF INJECTING 200, 400 AND 1000 SKIN-TEST DOSES AS SHOWN BY RETEST FIVE AND THIRTEEN MONTHS AFTERWARD AT ST DOMINICK'S HOSPITAL.

Retest at five months		%	Thirteen months %
One hundred	++ = 0 =	0.0	++ = 20 very susceptible
positive cases	+ = 8 =	9.8	+ = 9 susceptible
originally at St. Dominick's	± = 27 =	33.3	± = 14 doubtful
Test by	− = 46 =	56.8	− = 57 immune
Dr. Schroder			
	Total	81.0	Total 100

With greater knowledge, the doses were gradually increased.

We now inject 50,000 to 60,000 skin-test doses in five doses, namely 600, 2000, 6000, 20,000 and 30,000 skin-test doses. The Dicks report to us that they also give five injections and now give over 100,000 skin-test doses and they make the last dose 60,000 or even 100,000 and obtain a change from positive to negative in about 95 per cent. of the children, and that this remains for the great majority for a period of at least two years.

In contacts we give the first dose of the vaccine in one arm and the Dick test in the other. If the Dick test is positive the vaccine is continued.

The Therapeutic Use of Antiserum in Erysipelas.—This is used as in scarlet fever. It should be polyvalent for the main types of hemolytic streptococci occurring in erysipelas.

Antistreptococci Sera in Other Infections.—These are still in the experimental stage. The difficulty is due to the many types of streptococci causing infection.

Use of Toxins in the Treatment of Tumors.—Fehleisen inoculated cultures, obtained in the first instance from the skin of the patients with erysipelas, into patients in the hospital suffering from inoperable malignant sarcomata, and he obtained positive results. These experiments were undertaken on the ground that occasionally malignant tumors had previously been found to improve or entirely disappear in persons who had recovered from accidental erysipelas. This fact was therapeutically applied to the treatment of malignant tumors. Then the mixed toxins of the streptococcus and *B. prodigiosus* were given, and it became apparent that the toxins of the latter organism were much the more important. In some cases of inoperable sarcoma this method met with success.

Natural Immunity to Streptococci.—In the inflammations due to hemolytic streptococci we note a great difference in the tendency to the

production of immunizing and curative substances in the blood by an infection. The very important question of tissue specificity and local immunity of hemolytic streptococci has been studied in an interesting way by Gay and his associates.[1] For years they have been working with a single strain of hemolytic streptococcus isolated originally from a case of human empyema. They have greatly increased its virulence for rabbits by the pleural route (to 0.001 c.c.). If they give intradermally 0.1 c.c. of a twenty-four-hour broth culture of this passage strain they can produce erysipelas regularly, from which the rabbit recovers, although twice this dose leads to a fatal septicemia. Recovery from erysipelas protects an animal completely against reinoculation intradermally elsewhere on the body. It does not, however, protect the animal against intravenous inoculation with the same dose, which is the minimal lethal dose by this route. The converse they found also to be true.

On the other hand we have the interesting experiment of Koch and Petruschky. They inoculated cutaneously a man suffering from a malignant tumor with a streptococcus obtained from erysipelas. He developed a moderately severe attack, which lasted about ten days. On its subsidence they reinoculated him; a new attack developed which ran the same course and over the same area. This was repeated ten times with the same results.

The severe forms of infection, such as septicemia following injuries, operations and puerperal infections, show little tendency to be arrested after being well established. In these conditions the streptococcus poisons through its endotoxins rather than its exotoxins. The patient suffers from sepsis rather than toxemia.

Non-hemolytic Streptococci.—Non-hemolytic streptococci, most of which produce a green zone (methemoglobin) on blood agar plates, are not of such active virulence as are the hemolytic types. The majority of strains seem to be slowly invasive and may produce chronic inflammation of a low grade. The methemoglobin produced by many of the strains in variable amounts is probably a combination of reduction and oxidation processes (Heubner,[2] Cole[3] and Blake[4]). It has no relation to virulence and very little to carbohydrate reactions. Based on cultural and agglutinative reactions Krumwiede and Valentine[5] showed that strains of this group isolated from a series of human cases are practically heterogeneous.

Rosenow and his coworkers[6] claimed that a variety of non-hemolyzing streptococci is the cause of poliomyelitis, another of encephalitis (see Filtrable Viruses). We believe he is mistaken. Rosenow has published extensive reports of the localization of streptococci and of their taking on the power to produce lesions in special tissues. Tunnicliff

[1] Jour. Immunol., 1923, vol. **8**; Jour. Inf. Dis., 1922, **31**, 101; Jour. Am. Med. Assn., 1923, **80**, 1298; Jour. Inf. Dis., 1923, **33**, 338; Arch. Path. and Lab. Med., 1926, **1**, 590.
[2] Arch. Exp. Path. u. Pharm., 1913, **72**, 239.
[3] Jour. Exp. Med., 1914, **20**, 363.
[4] Ibid., 1916, **24**, 315.
[5] Jour. Inf. Dis., 1916, **19**, 760.
[6] Ibid., 1918, **22**, pp. 281, 313, 345.

claims that a green-producing streptococcus is the cause of measles. Ferry has also isolated a green-producing streptococcus from measles cases which he thinks is the cause. Each claims an antiserum as having protective qualities. We have not been able to convince ourselves either that a streptococcus is the cause of measles (see Filtrable Viruses) or that the antiserum made from injecting animals has any immunizing effect against measles.[1] Ferry's streptococcus makes an extracellular toxin which, injected in animals, stimulates an antitoxin. Various investigators in different parts of the world, particularly Poynton and Payne (1900), Beattie (1904), Small (1927), Birkhauk (1927), have isolated nonhemolytic streptococci from cases of rheumatic fever which they claim bear an etiologic relationship to the disease, but clear-cut evidence is lacking.[2] We have frequently isolated them from cases.

Bacteriological Identification.—Streptococci, using the name in a broad sense, can often be demonstrated microscopically by simply making a smear preparation of the suspected material and staining with methylene-blue solution or diluted Ziehl's stain. In order to demonstrate them microscopically in the tissues the sections are best stained by Kühne's methylene blue. In all cases, even when the microscopic examination fails, the cocci may be found by the use of special culture media.

To obtain them from a case of erysipelas a small amount of sterile broth may be injected into the skin at the margin of the erysipelatous area in which the cocci are most numerous and then withdrawn; part of it is transferred to ascitic or serum bouillon, part is streaked across freshly solidified agar in a Petri dish on which a drop of sterile rabbit's blood has been placed, and part is made up in dilution blood-pour-agar plates.

In septicemia the culture method is always required to demonstrate the presence of streptococci. For this purpose from 10 to 20 c.c. of the blood should be drawn from the vein of the arm aseptically by means of a hypodermic needle, and to each of three tubes containing 10 c.c. of melted nutrient agar kept at about 43° C. 1 c.c. of blood is added. After thorough mixing the contents are poured into Petri dishes. The remainder is added to several flasks containing 100 c.c. of nutrient broth, in order to produce a development of the cocci, which are found in small numbers in the blood. Clawson,[3] and later Cecil, Nicholls and Stainsby[4] recommended allowing the blood to clot, then storing in ice-box overnight, the next morning centrifuging and straining off all serum. The clot is then broken up and transferred to a 3-ounce bottle containing 50 c.c. of beef heart infusion broth. This is kept at 36° C. for five days before examining. The cocci can be best shown in blood by animal inoculation. Having withdrawn from the patient 10 c.c. of blood by means of a hypodermic syringe, under aseptic precautions, a portion of this is injected into the abdominal cavity of a mouse, while the other portion is planted into bouillon. Mice thus inoculated die from septicemia, chiefly by growth through the *lymphatics*, when virulent streptococci are present in only very small numbers in the blood. If a successful inoculation takes place we can, through the absence or presence of the development of capsules, often differentiate between the pneumococcus and the

[1] Jour. Am. Pub. Health Assn., 1927, **17**, 460.

[2] For a comprehensive review see D. and R. Thomson, monograph vol. **14**, part I of Ann. of Pickett-Thomson Research Lab., Williams and Wilkins Company, 1928.

[3] Jour. Inf. Dis., 1925, **36**, 444.

[4] Jour. Exp. Biol. and Med., 1928, **26**, 6.

streptococcus. Cultures may fail to do this. The development of a wide, clear zone about the colonies (upon blood agar), without a development of green pigment, indicates that the streptococci belong to the pyogenes group. The absence of a definite zone and the development of a green color indicate that they are pneumococci or streptococci of the viridans type. Colonies of pneumococci of Type V frequently produce large zones of hemolysis and may be thought to be streptococci. The growth in the Hiss inulin serum medium will generally differentiate between these two, as the pneumococci usually coagulate the serum, while the great majority of streptococci do not. The bile test should also be applied (p. 342). The detection of the streptococcus in the blood is an unfavorable prognostic sign.

The blood cultures in many cases of supposed septicemia give no results, for many of these cases develop their symptoms and even die from the absorption of toxins from the local infection, such as an amputation wound or an infected uterus or peritoneum, and the bacteria never invade the blood. When we get negative results we are, as a rule, utterly unable to test the case with curative serums with any accuracy, for the sepsis may be due to either the streptococcus, colon bacillus, staphylococcus, or some other pathogenic variety of microorganism.

In mixed infections when the streptococci present may not be isolated from original plates, plates made from an eighteen-hour culture in suitable broth may contain many isolated colonies.

The Relationship of Streptococci and Other Bacteria to Common Colds.—In connection with the great epidemic of influenza many studies of common colds were made throughout the world. In this country four groups of people worked under the general direction of a committee[1] composed of Rosenau (Boston), McCoy (Washington), Jordan (Chicago), and Park (New York). Besides these people Bloomfield and associates in Baltimore have made many investigations on this subject, and Olitzky and coworkers of the Rockefeller Institute have also made studies on common colds as well as on influenza.

It is difficult to summarize all of this work, but we may note some of the results obtained by us.[2]

The results of our studies indicate that of the different groups of microörganisms isolated by our procedure, all had the peculiarity that each group was an assemblage of many types. This was equally true for the pneumococcus, the influenza bacillus, the green-producing streptococci and the hemolytic streptococci. We obtained no evidence of the existence of a common filtrable organism. We accepted as the most delicate test of identity of types in strains the presence of specific agglutinable and agglutinogenic substances as determined by direct agglutination and agglutinin absorption methods. Biochemical characteristics are insufficient for such a determination; at most they divide a species only into the broader groups. In our isolation work, we are aware that the number of colony fishings may not have been enough in every group of bacteria to rule out a common strain. In a study of

[1] Started and largely financed by the Metropolitan Life Insurance Company, New York.

[2] Jour. Immunol., 1921, 6, 1.

case incidence alone, some groups may be ruled out as implicated in the epidemic; such as staphylococci, Gram-negative cocci and hemolytic streptococci. More work could be done on pneumococci, green streptococci, indifferent streptococci and some of the minority groups with a high case incidence. The question of the relationship between the bacterial types used in vaccination and the microbal strains obtained from the throats of the vaccinated remained mostly unanswered. The specific strains of organisms used in the vaccines were not found to any extent in either normals or the diseased.

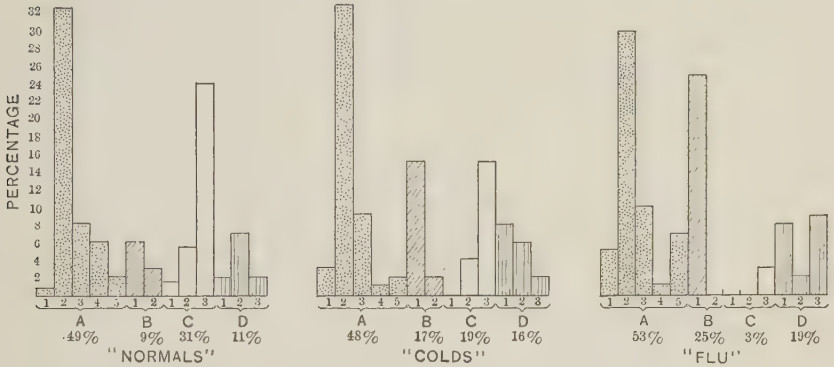


FIG. 104.—Chart showing comparative incidence of microörganisms by groups.

A = *Gram-positive cocci.*
1. "Hemolytic streptococci" (Smith and Brown's beta type).
2. "Green producing streptococci" (Smith and Brown's alpha type).
3. Pneumococcus group.
4. Staphylococcus group.
5. "Indifferent streptococci" (Brown's gamma type).

B = *Hemoglobinophilic bacilli.*
1. "Typical."
2. "Atypical."

C = *Gram-negative cocci.*
1. Meningococcus group.
2. Micrococcus catarrhalis group.
3. *M. flavus, M. siccus* and others.

D = *Other microörganisms.*
1. "Large Gram-negative bacilli."
2. *Bacillus mucosus* group.
3. "Others."

The evidence of immunological response to the vaccine was, as might be expected from the above findings, apparent only in the lessened incidence of pneumonia. The percentage of colds was as great among the vaccinated as among the unvaccinated. The pneumonia incidence was much less. The greater multiplicity of types of microbes believed to be capable of exciting common colds over those usually exciting pneumonia, is possibly the explanation of the apparent uselessness of the vaccines employed in this series in preventing minor respiratory infections while apparently affording considerable protection against pneumonia. It is

probably true that the persons in the vaccinated group had previously had more colds than those who did not avail themselves of vaccination. The results may have been somewhat more favorable than the outcome of the test suggests.

Comparative Incidence of Microörganisms from the Nasopharynx as Recorded in Our Original Records.—In trying to indicate the changes which we found in "normals," "colds" and "influenza" in the incidence of the group of microörganisms according to the chart headings, we obtained from our primary cultures the common incidence in each group by estimating the average percentages for each case on all the culture media used, adding these percentages in each individual group and dividing the sum by the total number of cases in that group. The above chart (Fig. 104) gives this common incidence in percentage for each group of cases.

CHAPTER XVI.

THE DIPLOCOCCUS OF PNEUMONIA (DIPLOCOCCUS PNEUMONIÆ, PNEUMOCOCCUS, STREPTOCOCCUS PNEUMONIÆ, MICROCOCCUS LANCEOLATUS).

The diplococcus of pneumonia was observed in 1880 almost simultaneously by Sternberg and Pasteur in the blood of rabbits inoculated with human saliva. In the next few years Talamon, Friedländer, A. Fränkel, Weichselbaum, and others subjected this microörganism to an extended series of investigations and proved it to be the chief etiological factor in the production of lobar or croupous pneumonia in man. The relationship of this organism to the streptococcus group is spoken of in the preceding chapter.

The outcome of the various investigations proved that the acute lung inflammations, especially when not of the lobar pneumonia type, are not excited by a single variety of microörganism, and that the bacteria involved in the production of pneumonias are also met with in inflammations of other tissues.

In any individual pneumonic inflammation it is also found that more than one variety of bacteria may be active, either from the start or by later addition to the original infection.

Morphology.—Typically, the pneumococcus occurs as spherical or oval cocci, usually united in pairs, but sometimes longer or shorter chains consisting of from three to six or more elements and resembling the streptococcus. The paired cells are usually pointed at one end—hence the name *lanceolatus* or lancet-shaped. When thus united the junction, as a rule, is between the broad ends of the oval, with the pointed ends turned outward; but variation in form and arrangement of the cells is characteristic of this organism, there being great differences according to the source from which it is obtained. As observed in the sputum and blood it is usually in pairs of lancet-shaped elements, which are surrounded by a capsule. (See Fig. 105). When grown in fluid culture media longer or shorter chains are frequently formed similar to those of certain streptococci. On solid medium containing serum or blood the organisms are often elongated into bacillary forms. These may be seen also in direct smears from the spinal fluid in pneumococcus meningitis. Pneumococci are divisible serologically into a number of types (see Determination of Types). One type differs from the others morphologically and culturally chiefly on account of the larger amount of capsular material developed by it. This type has been variously termed Streptococcus mucosus, Pneumococcus mucosus and pneumococcus Type III. (See Varieties of Pneumococci.)

The capsule is best seen in stained preparations from the blood and exudates of fibrinous pneumonia or from the blood of an inoculated animal, especially the mouse, in which it is commonly, though not always, present. It is seldom seen in preparations from cultures unless special media are employed, except with recently isolated strains of Pneumococcus mucosus. The significance of the capsule is not completely understood. It probably serves as a protective agent and is related to virulence. Our recent work[1] supports this opinion. Type III cultures, having especially abundant capsular material, had a much greater resistance to the action of protective antibodies than Type III culture having smaller capsules. Flagella are not present.

Staining.—It *stains* readily with ordinary anilin colors; it is not decolorized after staining by Gram's method. (For capsule staining methods, see under Stains.)

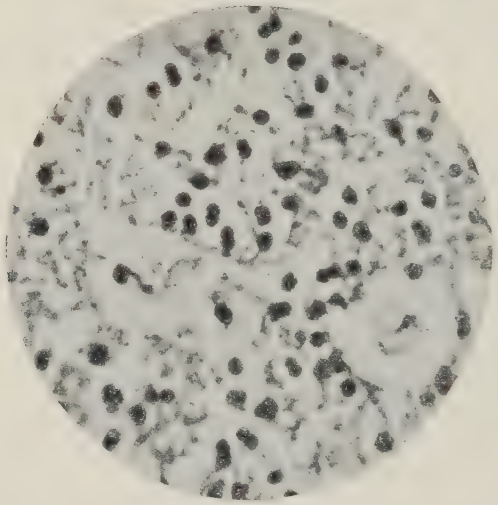


FIG. 105.—Diplococcus of pneumonia from blood, with surrounding capsule stained by method of Hiss.

Biology.—It grows equally well with or without oxygen; its parasitic nature is exhibited by the short range of temperature at which it usually grows—viz., from 25° to 42° C.—best at 37° C. In the cultivation of this organism neutral or slightly alkaline media should be employed (about pH 7.6 to 7.8). The organism when freshly isolated grows feebly on the serum-free culture media ordinarily employed for the cultivation of bacteria—viz., on nutrient agar and gelatin, in bouillon. The best media for its growth are given below.

Growth on Agar.—Cultivated on meat-infusion agar, after twenty-four to forty-eight hours at 37° C., the deep colonies are hardly visible to the eye. Under the microscope they appear light yellow or brown in color and finely granular. The surface colonies are larger, equalling

[1] Park, Bullowa and Rosenbluth: Jour. Am. Med. Assn., 1928, **91**, 1503.

in size those of streptococci, but are usually more transparent. If blood serum or ascitic fluid is added to the agar, the individual colonies are larger and the growth is more distinct, in consequence, and of a grayish color. The surface colonies are almost circular in shape under a magnification of 60 diameters, finely granular in structure and may have a somewhat darker, more compact center, surrounded by a paler marginal zone. With high magnification cocci in twos and short rows often distinctly separated are seen at the edges.

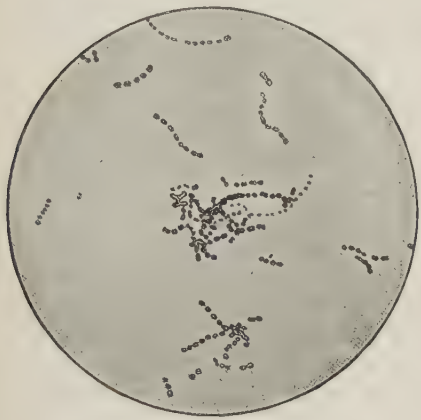


FIG. 106.—Pneumococcus from bouillon culture, resembling streptococcus.

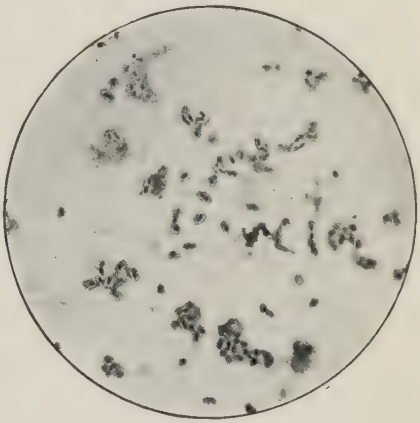


FIG. 107.—Pneumococci stained for capsule by Huntoon's method. (Huntoon.)

Growth on Blood Agar.—The colonies on blood agar are greenish with no definite zone of hemolysis (like colonies of Smith and Brown's alpha types of streptococci). There is a distinct checker-like or ringed edge. Methemoglobin is produced in broth cultures as shown on addition of freshly washed red corpuscle suspensions.

Growth on Blood Serum.—The growth on Löffler's blood-serum mixture is very similar to that on agar, but somewhat more vigorous and characteristic, appearing on the surface as a delicate layer of dew-like drops.

Growth in Bouillon.—In meat-infusion bouillon at the end of twelve to twenty-four hours in the incubator a slight cloudiness of the liquid will be found to have been produced. On microscopic examination cocci can be seen to be arranged in pairs or longer or shorter chains.

Growth in Milk.—It grows readily in milk, causing coagulation with the production of acid. Coagulation is not constant with some forms intermediate between the streptococcus and pneumococcus.

Growth on Gelatin.—The growth on gelatin in the usual way is slow, if there is any development at all, owing to the low temperature—around 20° to 24° C.—above which even the stiffest gelatin will melt. The gelatin is not liquefied.

Special Media.—When cultures are grown on serum-free media the vitality of some cultures may indeed be indefinitely prolonged; but after transplantation through several generations it is found that the cultures begin to lose in virulence, and that they finally become non-virulent. In order to restore this virulence, or to keep it from becoming attenuated, it is necessary to interrupt

the transplantation and pass the organism through the bodies of susceptible animals.

The vitality is prolonged and the virulence less rapidly lost if serum ascitic fluid or blood is present in the medium. (1) Serum or ascitic semisolid (stab cultures), a mixture of one-third human or animal blood serum or ascitic or pleuritic fluid and two-thirds bouillon, (2) nutrient agar streaked with human, horse, or rabbit blood, (3) pneumococcus broth (see under Media), (4) vitamin broth (see under Media) are the most satisfactory media for the growth and preservation of cultures.

Hiss Serum Water With or Without Inulin.—Inulin is fermented by typical recently isolated pneumococci with coagulation of the serum, while most streptococci fail to ferment the inulin. The addition of 1 per cent. of peptone to serum water is advisable, as with the plain serum water false negative results may be obtained. This fermentative reaction is of considerable value, but does not serve as an absolute criterion for separation from streptococci.

Calcium Broth With or Without Dextrose.—The addition of a small piece of marble to each tube of broth is the most satisfactory way of preparing it. Marble broth for this purpose was suggested independently by Bolduan and Hiss as very satisfactory for growth.

Action of Bile and Bile Salts.—If 0.1 c.c. of rabbit or ox bile be added to 0.3 c.c. of a broth culture of pneumococci, the culture becomes clear due to the dissolving of the cocci. A 10 per cent. solution of sodium taurocholate has the same action. Streptococci are not dissolved by bile. Serum and glucose interfere with the reaction. According to Sellards,[1] dilute sodium hydroxide helps the solubility in bile of certain strains of pneumococci.

Variations in Colony Morphology.—When pneumococci are plated on suitable media different types of colonies are found, as is the case with many other bacterial varieties. Young colonies of cultures freshly isolated from pneumonia cases or recently passed through susceptible animals are generally the "smooth" type, having a smooth, shiny surface, a rounded contour and a moist, thin consistency. Later they become flattened and have a checker-like appearance. Other cultures, especially those which have been cultivated on laboratory media for some time form colonies of the "rough" type, having a dull, slightly granular surface, a flat or checker-like contour and are more coherent, sometimes dry and brittle. Colonies of this general type are often very small after twenty-four hours' incubation. The "smooth" and "rough" types merge into each other and it is often difficult to decide to which category a culture belongs. In general, the more virulent (for mice) cultures conform to the "smooth" colony type, the less virulent and non-virulent to the "rough." However, strains of the former are sometimes comparatively non-virulent and some strains of the latter are virulent.[2]

Resistance to Light and Drying.—On artificial culture media the pneumococci tend to die rapidly. This is mainly due to the production of autolytic ferments. If growth is arrested at the end of eighteen to

[1] Jour. Am. Med. Assn., 1918, **71**, 1301.

[2] Griffith: Public Health and Medical Subjects, Ministry of Health, London, 1923, No. 18.

twenty-four hours by storing the culture at 15° C., they may remain viable and retain much of their virulence for months. In media containing a fermentable carbohydrate cultures are very rapidly destroyed by the acid produced. In sputum they live for a long time.

Pneumonic sputum attached in masses to clothes, when dried in the air and exposed to diffuse daylight, retains its virulence, as shown by injection in rabbits, for a period of nineteen to fifty-five days. Exposed to direct sunlight the same material retains its virulence after but a few hours' exposure. This retention of virulence for so long a time under these circumstances is accounted for by the protective influence afforded by the dried mucoid material in which the micrococci were embedded. Guarnieri observed that the pneumococci in the blood of inoculated animals, when rapidly dried in a desiccator, retained their virulence for months; and Foá found that fresh rabbit blood, after inoculation and cultivation in the incubator for twenty-four hours and subsequent storage in a cool, dark place, remained virulent for sixty days. There are many conditions, therefore, in which the virulence of the micrococcus is retained for a considerable length of time; the fine spray expelled in coughing and loud speaking that remains suspended in the air soon dries so completely that probably pneumococci rarely survive after two hours. The action of chemical disinfectants and of heat is given in Part III under Disinfection.

Attenuation of Virulence.—The loss of virulence which occurs when the micrococcus is transplanted through several generations in culture fluid containing no blood has already been referred to. Attenuation of virulence accompanied by serological and cultural changes associated with degradation takes place rapidly when cultures are grown in homologous antiserum.[1]

Restoration and Increase of Virulence.—The simplest and perhaps the most reliable method of restoring lost virulence for any susceptible animal is by passage through the bodies of highly susceptible animals of the same species. Apparently the method is successful only within certain limits, which are probably determined by the degree of degradation of the individual organisms in the culture. Growth in fresh blood also increases the virulence for the homologous animal.

Maintenance of Virulence.—This is best done by drying the spleen of an infected animal in a desiccator at freezing temperature. The spleen should be removed just before or immediately after death. The virulence may be preserved in this way for several months and probably longer.

Toxin Production.—We have little exact knowledge upon the nature of the poisonous substances produced by or through the growth of the pneumococci in animal tissues or artificial media. Attempts to show an exotoxin in culture filtrates have failed. Rosenow showed that the autolysis of virulent pneumococci in NaCl solution brings into the solution a group of substances which inhibits the action of the pneumo-

[1] Stryker: Jour. Exp. Med., 1916, **24**, 49. Griffith: Reports on Public Health Subjects, No. 18, Ministry of Health, London.

cocci opsonin. Such extracts are also poisonous in comparatively large doses for animals. Pneumococci dissolved in bile uniformly yield a toxic product.[1] A hemotoxin is present in the bacterial cell and is liberated by rupture of the cell.[2]

Weiss and Kolmer[3] have prepared an antigen with the bodies of the pneumococci which they say produces a specific intracutaneous reaction in patients in early stages of pneumonia. Bigelow[4] did not find it of practical use in advising treatment.

Precipitable Substance.—This is demonstrable[5] in broth cultures within four to six hours' incubation indicating that the substance may be excreted rather than due to autolysis of the cocci. This substance is demonstrable not only in lung lesion and sputum but also in the blood and in the urine. Its concentration in the urine and therefore its successful demonstration is relatively proportionate to the severity of the disease. Avery and Heidelberger[6] have found that there are two substances in the pneumococcus that are precipitable, one type specific, which is of a carbohydrate nature, and the other group specific, that is of a protein nature.

Occurrence in Man during Health.—It is probable that in crowded communities some one at least of the types of the pneumococcus is present on the mucous membranes of most persons. We have found it generally present not only in the throats of persons living in New York City,[7] but also in those of persons living on farms and in the Adirondack Mountains. It is commonly present only on the mucous membranes of the bronchi, trachea, pharynx and nostrils. The healthy lung seems to be generally free from it. The type found is usually one of the less virulent members of the heterogeneous group (see below). Carriers of the more virulent types also occur, usually due to contact with a case of pneumonia.

Pathogenicity in Man.—The following gives the conditions in which the pneumococcus is found and the frequency.

Netter found in 46 consecutive pneumococcus infections in children there were:

Otitis media	29 cases.
Bronchopneumonia and lobar pneumonia	13 "
Meningitis	2 "
Pleurisy	1 case
Pericarditis	1 "

The comparative number of the different infections varies according to the circumstances under which the cases were taken, such as the season of the year and the presence of other infectious diseases.

[1] Cole: Harvey Lecture, New York, 1913, Abs. Jour. Exp. Med., 1912, **16**, 644.
[2] Avery and Neil: Jour. Exp. Med., 1924, **39**, 745.
[3] Jour. Immunol., 1908, **3**, 395.
[4] Arch. Int. Med., 1922, **29**, 221.
[5] Dochez and Avery: Jour. Exp. Med., 1917, **26**, 477.
[6] Jour. Exp. Med., 1923, **38**, 81 and 73
[7] Report of Respiratory Commission, Department of Health, New York City, Studies on the Pneumococcus, Part I, Reprinted from Jour. Exp. Med., 1905, **7**, 401. Studies on the Pneumococcus, Part II, Reprinted from Jour. Infect. Dis., 1906, **3**, 774.

Avery and co-workers[1] found among 529 cases diagnosed clinically and pathologically as acute lobar pneumonia that the following were the etiologic agents demonstrated:

Diplococcus pneumoniæ	454
Streptococcus pyogenes	7
Bacillus influenzæ	6
Friedländer's bacillus	3
Staphylococcus aureus	3
Streptococcus mucosus	1
Mixed infection with combinations of Staphylococcus aureus, Friedländer's bacillus, B. influenzæ, St. pyogenes and St. viridans	6
Undetermined	49
Total	529

Pneumococci are present in about 95 per cent of cases of lobar pneumonia. Usually no other bacteria are obtained from the lungs. The more recent the infection the greater is the number of bacteria found in the diseased lung area. As the disease progresses, these decrease in number until finally at the crisis they have almost disappeared from the tissues, though at this time and long after convalescence they may be present in the sputum.

The pneumococci are found partly in the alveoli and bronchioles of the inflamed lung and partly in the lymph channels and blood capillaries. Most of the organisms are found free, but a few are found in the leukocytes. Through the lymph channels they find their way to the pleura and to adjacent lymph glands. From the capillaries they find their way to the general blood current, and thus to distant parts of the body.

A bacteriemia is probably present, at least in the earlier stages, in a large percentage of lobar pneumonias. In many instances this would be slight and, to demonstrate the presence of pneumococci, would require the culturing of large quantities of blood. Using 3 c.c. of blood, Rosenbluth obtained positive blood cultures in 35 per cent. of Type I infections, in 31 per cent. of Type II, in 25 per cent. of Type III and 7 per cent. in cases due to other than these types. In a general way the prognosis is grave in proportion to the numbers of organisms present in the blood. Some of the complications of pneumonia (see below) are due to localization of the blood-borne pneumococci in other organs or tissues. In a number of instances the fetus has been found infected.

Bronchopneumonia is frequently a secondary infection, complicating measles, influenza, etc., and bacteria other than pneumococci are often encountered. The pneumococcus is the causative agent in about one-half the cases; the other half are mostly due to the streptococci and influenza bacilli.

Inflammations Complicating Pneumonia.—In most cases of lobar pneumonia and frequently in bronchopneumonia pleurisy is developed, which is commonly excited by the same microörganism that was predominant

[1] Avery, Chickering, Cole and Dochez: Monograph of the Rockefeller Institute for Medical Research, New York, 1917, No. 7.

in the pneumonia. With pneumococci the exudate is usually moderate and of a fibrinous character, but may be more abundant and of a serofibrinous or purulent character. When the pleurisy is marked it is more apt to continue after the cessation of the pneumonia. Pleurisy due to pneumococci is more apt to go on to spontaneous recovery than that due to streptococci or staphylococci which may follow the pneumonias due to secondary infection.

The most frequent pneumococcic infections next to pleurisy, following a pneumonia, are those of the middle ear, pericardium, endocardium, and meninges, and these not infrequently arise together. Pneumococcic inflammations of the heart valves are apt to be followed by extensive necrosis and growth of vegetations. In these cases pneumococci can sometimes be found in the blood for many weeks. Pericarditis due to pneumococci is a frequent complication, but is usually not serious. Arthritis, periarthritis and osteomyelitis are rarer complications of a pneumococcic pneumonia. Besides moderate parenchymatous inflammation of the kidney, which occurs in most cases of pneumonia, well-marked inflammation may occur in which pneumococci exist in the kidney tissue in large numbers.

Presence in Inflammatory Process Not Secondary to Pneumonia.—It is now known that the pneumococcus may infect and excite diseases in many tissues of the body independent of any preliminary localization in the lung. As a rule these processes are acute and usually run a shorter and more favorable course than similar inflammations due to the streptococci.

The most frequent primary lesions excited by the pneumococcus, other than lobar pneumonia, bronchopneumonia and bronchitis, are probably meningitis, otitis media with its complicating mastoiditis, endocarditis, pericarditis, rhinitis, tonsillitis, conjunctivitis and keratitis; septicemia, arthritis and osteomyelitis; inflammation of the epididymis, testicles and Fallopian tubes; peritonitis, etc. The localization in the meninges and endocardium follows invasion of the blood stream, and a coincident bacteriemia or septicemia may be present.

Pneumococcic peritonitis and appendicitis are comparatively infrequent. The exudate is usually seropurulent.

Conjunctivitis due to pneumococci frequently occurs in epidemic form and is often associated with rhinitis.

The pneumococcus and streptococcus are the two organisms most frequently found in otitis media. The cases due to the pneumococcus are apt to run the shorter course, but have a tendency to spread to the meninges and cause a meningitis. The pneumococci may also find their way into the blood current. This usually follows after sinus thrombosis.

In bronchitis the pneumococcus is frequently met alone or in combination with the streptococcus, the influenza bacillus, or other bacteria.

In certain epidemics pneumococcic bronchitis and pneumonia may simulate influenza very closely and cannot be differentiated except by bacteriological examinations.

Primary pneumococcic pleurisy is frequent in children: it is very

often purulent, but may be serous or serofibrinous. Its prognosis is better than that in cases due to other organisms. Frequently we have streptococci and staphylococci associated with the pneumococci.

Pathogenesis in Lower Animals.—Most strains of the pneumococcus are moderately pathogenic for numerous animals; mice and rabbits are the most susceptible, indeed some strains are intensely virulent for these animals. Guinea-pigs and rats are much less susceptible. Pigeons and chickens are refractory. In mice and rabbits the subcutaneous or intracutaneous injection of small or moderate quantities of pneumonic sputum in the early stages of the disease, or of a twenty-four-hour broth culture from such sputum, or of a pure, virulent broth culture of the diplococcus, usually results in the death of these animals in from one to seven days. The course of the disease produced and the post-mortem appearances indicate that it is a form of septicemia. After injection there is loss of appetite and great debility. The postmortem examination shows a local reaction, which may be of a serous, fibrinous, hemorrhagic, necrotic or purulent character; or there may be combinations of all of these conditions. The blood of inoculated animals immediately after death often contains diplococci in very large numbers. For microscopic examination they may be obtained from the blood, and usually from pleural and peritoneal exudates that may be present.

True localized pneumonia does not usually result from subcutaneous injections into susceptible animals, but injections made through the thoracic walls into the substance of the lung may induce a typical fibrinous pneumonia. This was first demonstrated by Talamon, who injected the fibrinous exudate of croupous pneumonia, obtained after death or drawn during life from the hepatized portions of the lung, into the lungs of rabbits. Wadsworth showed that by injecting virulent pneumococci into the lungs of rabbits which had been immunized, a typical lobar pneumonia was excited, the bactericidal property of the blood being sufficient to prevent the general invasion of the bacteria. Pneumonia may be produced in dogs, and in rabbits less easily, by intratracheal injections.

Varieties of the Pneumococcus.—As among all other microörganisms minutely studied, different strains of pneumococci show quite a wide range of variation in morphology and virulence. These variations are so marked and so constant that they make it necessary to recognize several distinct varieties of the pneumococcus, and to class as pneumococci certain varieties which were earlier classed as streptococci—*e. g.*, the so-called Streptococcus mucosus capsulatus (Streptococcus mucosus Schottmüller)—when first isolated from pneumonic exudate or elsewhere, and planted on artificial culture media containing serum, grows as a rounded coccus with a small dense distinct capsule, principally in short or medium chains; it produces a large amount of mucus-like zoöglea, forming very large spreading colonies; it promptly coagulates fluid-serum media containing inulin. It is also very virulent for mice, but only moderately virulent for rabbits. After a number of culture generations on ordinary nutrient agar it apparently

loses some of these characteristics. It then grows in small colonies principally as naked diplococci which may be elongated and pointed, produces no zoöglea, and loses most of its virulence for mice and rabbits. It still coagulates inulin-serum media, and when transferred to serum media regains its former morphological characteristics. In 1905 Park and Williams showed that this variety should be placed with the pneumococci under the common name *pneumococcus mucosus,* because of the above characteristics and because it stimulated the production of specific antibodies that agglutinated in the higher dilutions only strains possessing these characteristics. They showed also that all of these strains fermented maltose. It was later classed by Avery and his co-workers as Type III of their agglutinative types.

We have found a remarkable difference in resistance to the action of protective serum between freshly isolated Type III strains from pneumonia cases and strains which have been maintained for some time on artificial media, even when the latter have such virulence that one to five diplococci injected intraperitoneally cause the death of mice in thirty-six hours. Apparently this is due to the greater development of capsular material by the freshly isolated strains. This phenomenon presents a difficulty in the development of efficient therapeutic antiserum for treatment of pneumonia due to this type.

Agglutination Reactions and Determination of Types.—That agglutinins are produced in animals by the injection of pneumococci was shown by Neufeld, Clairmont, and others. It has since been shown that the agglutination test may be used as a means of diagnosis. Neufeld,[1] Collins, Dochez[2] and Cole,[3] and others have shown that certain pneumococci may be grouped in types according to serum reactions. According to Cole and Dochez, the groups based upon their agglutination reactions are as follows: Types I and II, typical pneumococci; Type III, Pneumococcus mucosus. There remained about 40 per cent. which were not typed. These heterogenous strains were given the convenient name of Group IV. The last group includes many unrelated types which are dissimilar in their serological reactions, their relative virulence and other biological reactions. We are recommending that the term Group IV for heterogeneous strains be discontinued and that they be referred to simply as the heterogeneous group of strains or untyped pneumococci. One hundred and twenty strains of the heterogeneous group isolated from typical cases of lobar pneumonia during the season of 1928 were studied in this laboratory[4] and 70 of the strains, or 58 per cent., were classified in ten types. These are designated as Types IV to XIII, continuing the numbers from the well-known Types I, II and III. More recent work has shown that there are at least five other types among the strains which were left after this classification which

[1] Neufeld and Händel: Pneumococcus, Handbuch der pathogenen Microörganismen, Kolle and Wassermann, 2 Aufl., Bd. **4**.

[2] Jour. Exp. Med., 1912, **16**, 663.

[3] Arch. Int. Med., 1914, **14**, 56 (general discussion with bibliography).

[4] Cooper, Edwards and Rosenstein: Jour. Exp. Med., 1929, p. 461.

are present in sufficient numbers to warrant attention. These have been designated as Types XIV to XVIII. Statistics are still incomplete in regard to the prevalence and severity of these types. Avery[1] has shown that certain strains which had a delayed and usually an incomplete agglutination reaction with Type II antiserum were protected moderately by the standard Type II antiserum; on the contrary, antiserums stimulated by these strains did not protect to a perceptible degree against typical Type II strains. Employing monovalent antiserums, he divided these strains into two groups which he termed Sub-group II A and Sub-group II B. In our study we have classified these strains as separate types, our Type V corresponding to Sub-group II A and Type VI to Sub-group II B. We recommend this change because protection by specific antiserums against these groups is many times more effective than by the standard Type II antiserum, and treatment and diagnosis should be carried out with specific antiserums.

Certain strains studied by Sugg, Gaspari, Fleming and Neill[2] and by us were agglutinated in Type III antiserum to a marked degree, but less than typical Type III strains. With monovalent antiserums it was shown that these strains formed a separate group. There was some reciprocal cross protection between the typical Type III and the new group, but protection by specific antiserum against strains of the new group was so much stronger than by standard Type III antiserum that it seemed to us better to classify the group independently and we have called it Type VIII. Strains also have been encountered[3] which agglutinate in low dilutions with the three-type antiserums. Variations in the agglutinative characteristics of pneumococci can be induced, but rarely a change from one type to another. Griffith[4] claims to have induced a change of type by inoculating subcutaneously in mice degraded living cultures of one type with large masses of heat-killed virulent cultures of a different type. Miss Valentine, in this laboratory, in repeating this work, isolated virulent cultures corresponding to the heat-killed type from the heart's blood of some mice, but could only find the degraded type in the subcutaneous tissues. When variation is due to a simple degradation, passage through a susceptible animal often causes a return of its type reaction.

The following table by Cole gives the types he found in pneumonia in a series of cases occurring in New York City, and the relative mortality of the cases according to the infecting type:

Infecting type.	Cases.	Strain, per cent.	Deaths.	Mortality, per cent.
I	34	47	8	24
II	13	18	8	61
III	10	14	6	60
Not typed	15	21	1	7
Total	72	100	23	32

[1] Jour. Exp. Med., 1916, **24**, 7, 25.

[2] Ibid., 1928, **47**, 917.

[3] Blake: Jour. Exp. Med., 1917, **26**, 67; Clough: Bull. Johns Hopkins Hosp., 1917, **28**, 306.

[4] Jour. Hyg., 1928, **27**, 113.

120 strains from lobar pneumonia occuring in adults in New York City in 1928 not belonging to Types I, II or III were classified by Cooper as follows:

Infecting type.	Cases.	Strain per cent.	Deaths.	Mortality per cent
IV	19	16	7	37
V	6	5	5	83
VI	7	6	2	29
VII	11	9	2	18
VIII	4	3	1	25
IX	6	5	1	17
X	5	4	3	60
XI	4	3	1	25
XII	4	3	2	50
XIII	4	3	0	0
Not typed	50	43	10	22
Total	120	100	34	29

In different localities, and at different times in the same locality, the type percentage varies greatly and the mortality also varies somewhat. Reports of the many pneumonias recovering during the late war show a distinct difference in percentage of types. Lister, in South Africa, found a type distinct from Types I, II or III to be the prevailing type.

During the influenza epidemic of 1918 Types I, II and III were encountered in only 40 per cent. of the cases of pneumonia. The incidence of these types gradually increased in the following winters and after several years the usual incidence was again established.

Wollstein and Benson[1] found that pneumococcus other than Types I, II and III occurred more frequently in the pneumonias of young children and that the mortality due to this group was high (40 per cent.). Dr. Raia obtained the following percentages in 150 cases of lobar pneumonia at Bellevue Hospital in the winter of 1928–1929:

PNEUMONIA CASES IN YOUNG CHILDREN.

(Bellevue Hospital, November 1, 1928, to April 25, 1929. From cases studied by Drs. Raia and Plummer.)

Organism.	No. of cases.	Deaths.
I	12	1 (Lobar pneumonia).
II	2	
III	3	1 (Meningitis, meningitis and bronchopneumonia).
IIIa	2	
IV	4	1 (Bronchopneumonia).
V	7	1 (Lobar pneumonia and empyema).
VI	17	4 (2 bronchopneumonias and 2 lobar pneumonias).
VII	5	2 (Bronchopneumonias).
VIII	1	
IX	7	

Recently we found that an epidemic of colds, bronchitis and pneumonia of over 100 cases among children in an institution was caused by Type V. Only a limited number of observations are available on the

[1] Am. Jour. Dis. Child., 1916, **12**, 254.

groups occurring in meningitis. Valentine gives the following series: I, 2 cases; II, 7 cases; III, 2 cases; Group IV, 2 cases. Total, 13.

Pneumococci taken from normal throats or from slight inflammation fall usually into the miscellaneous group of heterogeneous strains. The strains belonging to Type I are widely scattered, being found not only throughout the United States but also in Europe.

Bacteriological Diagnosis.—Direct smears may be prepared from the sputum or other exudates and stained with the Gram and with the capsule stain. The microscopic findings may be verified by preparing "pour or streak" blood plates, isolation of pure cultures and determination of bile solubility. Isolation may also be made by intraperitoneal mouse inoculation (see p. 352). As a septicemic infection usually develops, the presence of the typical pneumococci is easily demonstrable in the heart's blood, from which cultures can then be isolated. In the pneumonias the probable etiological agent is determined by washing a kernel of sputum with sterile saline to remove the contaminating mouth bacteria and determining the preponderating variety of bacterium in stained smear. Blood agar plate cultures and subsequent isolations are used for verification of the morphological findings.

Rapid Methods for the Determination of Type of Pneumococcus.—*Sputum.*—The sample should come from the deeper air passages and be as free as possible from saliva and naso-pharyngeal mucus. Two methods are available: (*a*) Demonstration of "precipitable substance" in sputum, and (*b*) "mouse inoculation method." Before attempting any of these tests there should be available at least (see above) antisera for Types I, II and III which have been tested for presence of cross-reactions and minimal dilutions determined for specific testing. The less cross-action there is the better are they suited for this purpose as lower dilutions may be used, which leads to quicker reactions. It should be remembered that time is a compensating factor in group reactions. Thus, the specific reaction may appear at once or very quickly, and if sufficient time be allowed to elapse the degree of group action may obscure the result. The tubes should be observed at once and frequently thereafter. Some recent complaints of inaccuracy in the following methods are seemingly due to this factor, the tubes being observed only after one set time, thirty minutes.

Precipitable substance in the sputum is most easily determined by the coagulation method of Krumwiede and Valentine. The technic of the test is as follows:

"From 3 to 10 c.c. of the sputum, depending on the amount available, is poured from the sputum container into a test-tube. This is placed in boiling water for several minutes or longer until a more or less firm coagulum results, which will occur if the specimen is a suitable one. The coagulum is then broken up with a heavy platinum wire or glass rod, and saline is added. Just enough saline should be added so that, on subsequent centrifugalizing, there will be sufficient fluid to carry out the test. If too much is added, the resulting antigen may be too dilute. In some instances little or no saline is necessary, as sufficient fluid separates from the coagulum.

"After the addition of the saline, the tube is again placed in boiling water for

a few minutes to extract the soluble antigen from the coagulum, the tube being shaken several times during the heating. The broken clot is then thrown down by centrifuge, and the clear supernatant fluid used for the test. To hasten the appearance of the reaction and to obtain a reaction even should the antigen be dilute, we float the antigen over the 'type' serums, using the latter undiluted. Two-tenths c.c. of each of the 'type' serums are placed in a narrow test-tube, and the antigen added from a capillary tube with a rubber teat. If the tubes containing the serum are tilted and the antigen dropped slowly on the side of the tube just above the serum, no difficulty will be encountered in obtaining sharp layers, as the undiluted serum is sufficiently higher in its specific gravity. The tubes are then placed in the water-bath at from 50° to 55° C. and observed after several minutes.

"If a fixed type was present in the sputum, and should the sputum have been rich in antigen, a definite contact ring is seen in the tube containing the homologous serum. With sputums less rich in antigen, the ring may develop more slowly, and will be less marked. Some experience is necessary in detecting the less marked rings and in differentiating them from an apparent ring which may be confusing, if one of the serums is darker in color, giving thus a sharper contrast with the supernatant antigen. The true ring is more or less opaque, and this quality can be seen by tilting the tubes and looking at the area of contact against a dark background, for example, the lower edge of a dark shade raised to just above the level of the eyes. The advantage of the ring test is that a ring may be evident, whereas definite clouding or visible precipitate may appear only after longer incubation, or may be so slight even after an hour's incubation as to leave one in doubt. It is well to shake the tubes after twenty minutes, as many of the specimens will show definite clouding or precipitate either at once or on further incubation, thus checking the ring reading. A positive reaction usually appears within one-half hour and the majority are evident in less than ten minutes." Mucoid sputums give difficulty as the mucus dissolves in the extract and the test fluid becomes opalescent.

The mouse inoculation method is as follows:

A bean-sized mass of the sputum, about 1 c.c., preferably washed and emulsified, is injected intraperitoneally into a white mouse. The pneumococci multiply rapidly in the mouse peritoneum. After five to eight hours the mouse will usually show evidence of illness if one of the more virulent types was present. Puncture and withdrawal of some of the peritoneal exudate and examination of stained smears of this exudate may be employed as an indication whether the mouse should be killed or not. With sputums containing large numbers of virulent pneumococci less than five hours may suffice to yield an exudate rich enough in pneumococci for the test. Very mucoid sputums are apt to give difficulty in the precipitin test if sufficient time is not allowed for absorption of the mucus. The mouse is then killed, the peritoneal cavity opened and the exudate washed into a sterile Petri dish with 1 to 2 c.c. of saline, using a pipette. A smear taken from the exudate will indicate the presence or absence of other organisms. The peritoneal washings are centrifugalized at low speed to throw down the cells and fibrin. The supernatant fluid is transferred to another tube and centrifugalized at high speed until the fluid is clear. This is carefully removed. One may then proceed, utilizing this clear fluid for the precipitin test or one may resuspend the sedimented cocci in saline (density of eighteen-hour broth culture) and utilize this for the agglutination. The precipitin method is especially valuable where other bacteria are present, even with a mixture of a fixed type and heterogeneous varieties of pneumococci. The bacillus of Friedländer and the influenza bacillus are the types most likely to "come through" the mouse. For the precipitin method not more than the amount of saline noted should be used, as otherwise

the antigen may be too dilute to give a reaction. Where the agglutination method only is used this is not important, as the cocci only are used.

Sabin[1] has developed a microscopic agglutination method, observing agglutination in stained smear, which method has distinct advantages over the tube method. Three or four hours after injection some of the peritoneal exudate is obtained by puncture. Small drops are placed on a slide. To one is added a loopful of saline, to the others "type serums" in dilution of 1 to 10, the mixtures being spread thinly, so that they dry rapidly, and the spreads stained with fuchsin (10 c.c. saturated alcoholic solution plus 90 c.c. of water). A positive reaction is evidenced by stained clumps of diplococci. Clumping due to other than specific reactions are excluded by comparison of the saline and series of smears containing serum. As the mouse is not killed, the test may be repeated should the numbers of pneumococci be too small to give a satisfactory test. As fewer organisms are needed for this test, two outstanding advantages are obtained as compared with the test-tube method—speed and the possibility of using serums for many or all of the recognized types. Where sputums are not procurable swabbing of the throat may be used to procure material. In infants a catheter passed into the esophagus may be of use to obtain some of the swallowed sputum.

The above methods are reliable in direct proportion to the care taken in obtaining the sample of sputum. The success of the coagulation method depends on the content of coagulable substance which will be greater the more nearly the sputum is really a pulmonary exudate and the degree of pulmonary inflammation. In the very early stages of inflammation it is more likely to give negative results. Even when satisfactory coagulation occurs a negative reaction does not exclude an infection by a fixed type, as very little antigen may be present. With good samples, the method will give from 70 to 80 per cent. positive tests in Types I, II and III infections, thus allowing prompt use of the specific serum if indicated.

The mouse method will give a much higher percentage of positive results, as even the presence of contaminating heterogeneous varieties may not vitiate the test as the more virulent fixed types usually will come through in preponderance sufficient for test purposes, even though originally present in small numbers. For statistical purposes the above methods should be checked by isolating pure cultures from the sputum or preferably from the mouse heart for verification tests with the immune sera. The peritoneal exudate is not as satisfactory for check purposes as a mixed culture may be present, whereas the heart's blood is more likely to contain only the more virulent or invasive type.

It should be appreciated that the mouse findings do not necessarily establish the etiology of a pneumonia. The definite pneumococcus types usually are found only in pneumonia or other forms of inflammation of the respiratory tract. However, carriers do occur, most frequently of the Type III. The miscellaneous group is usually present in normals, and these may be more virulent for the mouse than some other bacterium which is actually causing the pneumonia. However, in the presence of definite lobar pneumonia the mouse method has high practical accuracy, especially in relation to the selection of antiserum

[1] Proc. Soc. Exp. Biol. and Med., 1929.

for therapy. It is of less practical accuracy in bronchopneumonia. A complete determination of etiology would require smear and cultural examination of the sputum. Lung puncture has also been employed to obtain material for studies in etiology.

Urine may be utilized for the determination of the infecting type. This method is of less value, however, because the demonstrable amounts of precipitable substance appears in only about 65 per cent. (usually the more severe cases), and as a rule the sputum examination is applicable earlier. The test is as follows:

Clear urine by centrifuge and set up precipitin reaction as above. To concentrate the antigen acidify 25 c.c. of urine by the addition of a few drops of acetic acid and boil down to 5 c.c., filter and add to 8 to 10 volumes of 95 per cent. alcohol, collect the precipitate that forms (by centrifuge), dissolve in 2 c.c. of saline, clear by centrifuge and use clear fluid for precipitin test.

Cerebrospinal fluid and other exudates will frequently give a precipitin reaction, utilizing the clear fluid obtained by centrifuge. The sediment may be utilized for mouse inoculation.

Immunity.—Following an attack of pneumonia some immunity is established, but this lasts only a short time. After successive injections of gradually increasing doses of virulent pneumococci into certain animals (horse, sheep, goat, rabbit), a serum of high protective and some curative power in experimental animals is obtained. The mode of action of this serum is still the subject of study. According to Wright, Neufeld, and others, its activity is due primarily to the opsonins. The mechanism of the crises in pneumonia is not understood, but the presence of increased opsonins and other protective substances at this time suggests that it is a phenomenon dependent upon the increase of these substances and associated cellular activity.

Pneumococcus Vaccine.—Only a limited number of cases have been treated under known conditions of specific relationship to the infecting pneumococcus. Favorable results have been reported by some observers. Similar favorable reports have been made by those utilizing stock vaccines. On the whole, there seems to be some indication that vaccine may influence the clinical course of the disease in some cases. Whatever benefit may be obtained is most probably due to non-specific elements. The dose may be from 5,000,000 to 50,000,000.

Vaccines have been used for prophylaxis with considerable success, but the drawback is the relatively short immunity which results and the fact that we cannot give a vaccine against all the types. Lister determined the dominant types in the area where he was working (South Africa) and prepared his vaccine accordingly. During a period of nine months following the vaccination not one of 11,000 vaccinated developed a pneumonia due to pneumococci of the types contained in the vaccine. Eighty-two cases due to other types did, however, develop. Cecil and Austin[1] obtained similar results with a vaccine containing Types I, II and III. They vaccinated over 12,000 men. Their results are difficult

[1] Jour. Exp. Med., 1918, **28**, 19.

of interpretation, as they obtained not only a decrease in pneumonias due to the fixed types, but a proportionate decrease in pneumonia due to the other types grouped together as Type IV, and also in those due to the Streptococcus viridans and Streptococcus hemolyticus. This apparent non-specific protection has no experimental parallel and we do not believe that one is justified in believing that the fixed types will give cross-protection in man when all animal experimentation is negative. The results as they stand are suggestive but by no means conclusive. Our own observations indicate that three injections of Type I pneumococcus vaccine develop a considerable immunity against Type I infections and that this lasts for about six months. The immunity following injections of Types II and III is probably somewhat less in amount. We do not believe that it is wise to urge general vaccination.

Dosage.—Saline vaccines, 6 to 9 billions of each type, divided into 3 to 4 doses five to seven days apart. Type III has been employed in relatively smaller doses than the other two types.

Ross has shown that the feeding of one dose of the filtrate of pneumococci dissolved in bile or of dead pneumococci to rats and to man will result in the development of antibodies.

Serum Therapy.—The experimental work in animals and the observation of cases has led to the general use of larger injections and the substitution of the intravenous for the subcutaneous method. The size of the patient and the strength of the serum in antibodies should be considered in controlling the size of the dose. The serum should be standardized, as in the case of antitoxins. Type I serum containing 200 to 500 units per cubic centimeter and Type II serum, 100 to 400 units per cubic centimeter, can be easily prepared or preferably concentrated refined antibody of four to five times this strength. By selecting horse serum of the highest potency antibody preparations containing 5000 to 10,000 units per cubic centimeter can be secured.

Every patient should receive as soon as possible 10,000 units of Type I and Type II antibody. If no outward reaction develops, a second dose should be given in two to three hours. If potent antibody becomes available for Type III and the new types it would be desirable that the same quantities of units for them should be given. As long as the temperature remains high, or septicemia is present, the injections of 10,000 to 20,000 units, according to the severity of the case, should be continued every six to twelve hours. When the case has been typed monovalent serum may be substituted, at least in part, for the polyvalent one. If the pneumonia is shown to be due to a type for which specific antibodies are not present in the serum, the injections should be discontinued, since the cross protection between types is so feeble that the serum would probably be of little use, and at present it is desirable to conserve the supply of serum.

A study[1] of the presence of antibody in the blood of patients before

[1] Transactions of Section on Pathology and Physiology of American Medical Association, 1927.

and after injections of antibody solution showed that in the great majority of cases due to Type I and in the majority of Type II the dosage indicated was sufficient to secure an excess of protective substances in the patient's blood. A few very severe cases of Type II showed an excess of toxic substance in the blood which was not neutralized by treatment and has been shown by careful estimations to require a larger amount of antibody than is practicable to give. Fortunately such cases are few.

The repetition of the dose every six to twelve hours is founded on the desire to give it sufficiently often to keep up an excess of antibody. The degree of disappearance after repeated injections is proportional to the severity of the infection. When antibodies begin to persist after an injection recovery usually follows. In our own experimental work we have found that a single dose of 0.1 c.c. protects a mouse for the next four days from an injection of 1000 fatal doses of living pneumococci, and as late as the fifteenth day, from 100 fatal doses. The mouse differs from the sick person in not having an infected area and associated free antigen in the blood and other body fluids.

The Results of Intravenous Injections of Specific Serum in Type I Infections.—When the unrefined serum is used there is in about 30 per cent. of the cases an almost immediate more or less severe chill with a considerable rise of temperature which lasts for a short period. This occurs usually after the first but sometimes after the later serum injections[1]. With the use of the refined antibody solution, as prepared by Felton or Banzhaf, less than 10 per cent. have chills with the first dose and almost none have chills following the later injections. If the blood contains pneumococci they disappear within twelve hours after the injection. The temperature usually rapidly falls after the initial rise to a point lower than before the rise and the symptoms, as a rule, improve sooner than the average untreated case of equal severity. The mortality up to the present time has been reduced by the serum at least 50 per cent. in the Type I cases and slightly in the Type II cases. Serum sickness with rashes, painful joints, swelling of lymph glands and other symptoms occur to a greater or less extent in about 50 per cent. of the cases getting the unrefined serum during convalescence. The serum sickness, while it lasts, is very annoying, but not dangerous. Injections of refined serum are not, as a rule, followed by serum sickness. The combined results[2] of the cases treated at Bellevue Hospital under the direction of Cecil and Plummer, at the Harlem Hospital by Bullowa and Rosenbluth, and at the New York Hospital by Baldwin, are seen in the following table:

[1] Jour. Am. Med. Assn., 1928, **90**, 1349.
[2] Ibid., **91**, 1503.

DEATHS PER HUNDRED CASES IN PATIENTS WITH LOBAR PNEUMONIA TREATED WITH SERUM AND WITHOUT SERUM.

(Experience of Bellevue, Harlem and New York Hospitals, 1927–1928.)

Type.	Treated with serum.			Not treated with serum.			Difference in case fatality (A–B).
	Cases.	Deaths.	Deaths per 100 cases.	Cases.	Deaths.	Deaths per 100 cases.	
Type I:							
Combined experience . .	266	51	19	249	82	33	14
Bellevue Hospital . . .	144	27	19	132	43	33	14
Harlem Hospital . . .	114	23	20	109	37	34	14
New York Hospital . .	8	1	13	8	2	25	
Type II:							
Combined experience . .	176	61	35	165	74	45	10
Bellevue Hospital . . .	107	42	39	95	50	53	14
Harlem Hospital . . .	61	18	30	63	20	32	2
Type III:							
Combined experience . .	82	27	33	92	27	29	4
Bellevue Hospital . . .	32	12	38	52	14	27	11
Harlem Hospital . . .	47	15	32	38	12	32	0
New York Hospital . .	3	..	..	2	1	50	
Miscellaneous types:							
Combined experience . .	313	76	24	324	83	26	2
Bellevue Hospital . . .	131	39	30	128	51	40	10
Harlem Hospital . . .	171	35	20	190	28	15	5
New York Hospital . .	11	2	18	6	4	67	

Chemotherapy.—Morgenroth and Levy[1] showed that ethylhydrocuprein (optochin), a derivative of hydroquinone, was of value in the treatment of experimental pneumococcus infections. This substance has an almost specific action on pneumococci and *in vitro* its greater action on the pneumococcus group serves to separate this group from the related cocci.

It acts even in dilutions as great as 1 in 1,000,000. It has a protective and curative action in guinea-pigs and mice infected with pneumococci. Small doses of optochin increase considerably the protective power of the type homologous antipneumococcus serum in experimental animals (Moore).

The pneumococcidal action of the serum after administration of the drug was studied in rabbits by Moore[2] and in patients suffering from acute lobar pneumonia by Moore and Chesney.[3]

The use of the drug in humans may give rise to amblyopia or amaurosis which is generally transitory if the drug be discontinued when these symptoms appear. Moore and Chesney have collected from the literature 786 cases of lobar pneumonia treated with the drug, among which the mortality was 12.84 per cent.; the eye symptoms referred to above occurred in 4.4 per cent. of the cases and in one of these this disturbance was more or less permanent.

[1] Berl. klin. Wchnschr., 1911, **47**, 1560, 1979.
[2] Jour. Exp. Med., 1915, **31**, 389, 557.
[3] Arch. Int. Med., 1917, **19**, 611.

CHAPTER XVII.

MENINGOCOCCUS (MICROCOCCUS (INTRACELLULARIS) MENINGITIDIS OR NEISSERIA INTRACELLULARIS) AND THE RELATION OF IT AND OF OTHER BACTERIA TO MENINGITIS.

While certain microörganisms are respectively the cause of isolated cases of primary meningitis (see below) the meningococcus is the most frequent etiological agent of purulent meningitis either sporadic or endemic. Intracellular Gram-negative diplococci were first described as occurring in meningitis by the Italians, Marchiafava and Celli, in 1884, but the organisms were not isolated and cultivated until 1887 when Weichselbaum studied their characteristics and showed that they were clearly distinguishable from the pneumococcus. He called the organism Diplococcus intracellularis meningitidis. In 1895 Jaeger and Scheurer drew especial attention to the etiological relationship of the organism to the epidemic form of cerebrospinal meningitis. They also believed that in cases of primary meningitis the cocci found their way to the meninges either directly from the mucous membranes of nasal passages or sinuses through the lymph channels or through the blood stream. The nasal secretion of carriers is dangerous to the community because carriers are not recognized as such and mingle freely with the general public. Only a minority of those exposed to infection develop the disease. The fact that they do not contract the disease indicates that they have at least a certain amount of immunity. Epidemics are most prevalent in winter and spring, a time favorable to influenza and pneumonia and other diseases where the respiratory mucous membrane is the site of infection.

Morphology.—This organism occurs as kidney-shaped micrococci, usually united in pairs, but also in groups of four and in small masses, sometimes solitary, and small degenerated forms are found. It has no well-defined capsule. Culture forms resemble strongly those of gonococci, but, on the whole they are much more irregular in size especially as they grow older. In the sediment from spinal fluids (see Fig. 108) meningococci are both intra- and extracellular. When they are intracellular the prognosis is better, and frequently in cases where they are extracellular at first they later become intracellular.

Staining.—They stain with all the ordinary anilin dyes and are readily decolorized by Gram's solution. A smear from a culture shows characteristic irregularity in staining, some of the cocci taking the counterstain poorly and some staining deeply.

Cultivation.—They grow between 25° and 38° C., best at about 35° C. They are easily isolated on 2 per cent. glucose ascitic agar neutral to phenolphthalein. We have found that the so-called "vitamin" agar with the addition of blood is one of the best mediums for direct isolation. They are kept well on semisolid media. The liver medium recommended by Dopter is an excellent one for stock transplants. (See chapter on Media.) Different strains of meningococci vary markedly in the ease with which they may be cultivated.

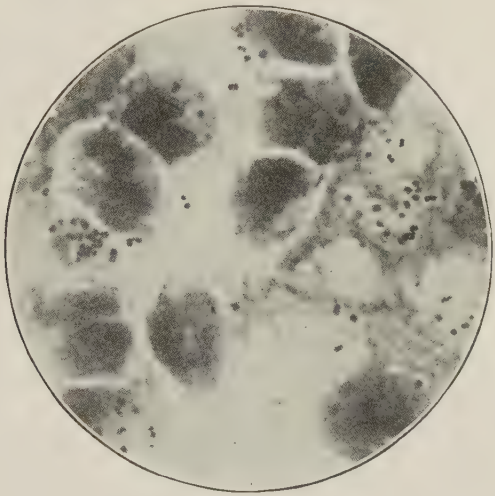


FIG. 108.—Diplococcus intracellularis meningitidis in pus cells. × 1500 diameters. (Neal.)

The colonies on serum agar directly from spinal fluid are convex, slightly flattened, smooth, moist and shiny. The colonies of recently isolated strains develop to 1.5 to 2 mm. after twenty-four hours' incubation and have the same appearance as above. Microscopically, the colonies have a smooth margin and show fine granulations. After cultivating for some time on artificial medium, they may become smaller, almost pin-point in size, dry and rough surfaced, with a serrated margin and coarse granulations showing microscopically. This may be an indication of degradation (see Agglutination), other evidences of which may also be found. In some cultures a mixture of typical and degraded forms may be found. In forty-eight hours the great majority of the strains develop in the center of the colonies, the crystals described by Elser and Huntoon.[1] Gonococcus colonies may also develop similar crystals but not in such great abundance. On blood agar or serum agar the growth is much more luxuriant than on plain agar and the colonies are larger than the gonococcus. Cultivated in artificial media, while it often lives for weeks, it may die within four days, and requires, therefore, to be transplanted to fresh material at short intervals.

Resistance.—The meningococcus is readily killed by heat, disinfectants (see table under Disinfection), sunlight and ordinary drying. Dried rapidly under freezing temperature and kept frozen, it may remain alive for years (Elser and Huntoon).

[1] Jour. Med. Res., 1909, **20**, 377.

Agglutination.—Different strains vary much in their agglutinability. The agglutination test is utilized as a routine in the identification of cultures from other sources than the cerebrospinal fluid. The slide agglutination method, using a 1 to 10 or greater dilution of immune horse serum (the dilution depending on preliminary tests with known strains) and a similar dilution of a normal horse serum as a control, is a great convenience in examining the colonies from plates, when searching for carriers.[1] The drops after drying are used for control by staining with the Gram stain. The macroscopic tube agglutination may also be employed, using agar cultures isolated from the colonies. The latter method is less expedient and in practical work seems to have no advantage over the slide method except possibly for verification purposes. In these tests a serum of a horse immunized with the dominant varieties of meningococci is utilized. It is evident that such a serum, because of group reactions, may give a positive reaction with Gram-negative cocci which are not meningococci. With our present lack of knowledge as to the limits of the meningococcus group, they must be considered meningococci or at least potential agents for the production of meningitis. We are unable therefore to identify all meningococci with surety. If, however, a strain can be identified as belonging to one of the four dominant groups (see below), we can definitely say it is a true meningococcus. As varieties, not belonging to these groups, are encountered in meningitis, the agglutination reactions are of no value in indicating the disease-producing capacity of such varieties.

Agglutination Groups of Meningococci.—Elser and Huntoon,[2] Wollstein[3] and others found differences in the serum reactions of strains. The results indicated a tendency to grouping but were apparently not sufficiently constant or clean-cut to warrant a definite separation. Dopter found that certain strains differed rather sharply both agglutinatively and absorptively from the general type and suggested the term "parameningococcus" for such aberrant varieties. Gordon[4] has extended the use of the method of agglutinin absorption and believes that four distinct types (groups?) exist as well as an apparently heterogeneous group. Classifying strains isolated from epidemic cases among the troops, he has found that the majority of the strains coming from meningitis cases fell into four main groups. He numbers his types I, II, III and IV. Types I and III and Types II and IV are related. Tulloch[5] repeated Gordon's findings, but his results indicated the existence of subvarieties of Type II. We have obtained similar results, but believe that group rather than type is more applicable because the strains are very closely related rather than identical in each division.

We have found that in interepidemic cases the dominant groups are

[1] Krumwiede: Jour. Am. Med. Assn., 1917, **69**, 358.

[2] Elser and Huntoon: Jour. Med. Res., 1909, **20**, 377. Griffith: Jour. of Hyg., 1915–17, **15**, 446; Griffith: Jour. of Hyg., 1918, **17**, 124. Nicolle: Ann. de l'Inst. Past., 1918, p. 154.

[3] Wollstein: Jour. Exp. Med., 1914, **20**, 201.

[4] Jour. Royal Army Med. Corps, 1915, **25**, 411, also Jour. Hyg., 1918, **17**, 290.

[5] Ibid., 1912, **29**, No. 1. See also Dopter et Pauron: Comptes Rend. de la Soc. de Biol., 1914, **77**, 157.

less frequent. However, in the recent rise of meningitis in New York (1928) about 80 per cent. of the strains fell into Group I. It would seem that in this country the dominant, more-clearly defined types are the virulent strains which give rise to epidemics. One point should be emphasized, viz.: that the direct agglutination with specific type sera is only of relative value in determining the group to which a culture belongs. Even marked or complete cross-agglutination may be noted with two strains having distinctly different agglutinin absorptive capacities. Even immune sera from the rabbit will usually show these marked cross-reactions.

The question of determining the existence of types of meningococci, especially dominant types, and their relative prevalence is of the greatest importance in the production of therapeutic serums. How far cross-agglutination is associated with cross-protection is an important factor concerning which we have little laboratory experimental evidence. Clinical observations indicate, however, that an antiserum, potent against the prevailing dominant types, will have considerable curative power against the other less well-defined types.

Pathogenesis.—This organism has a low grade and very variable pathogenicity for laboratory animals. Following an intraperitoneal injection death may take place in from ten to forty-eight hours, dependent on size of dose and relative virulence of the bacterium. At autopsy there is fluid exudate into the abdomen and sometimes into the pleural cavity, congestion or hemorrhage of the adrenals, hemorrhages into the mesentery, central tendon of the diaphragm and into the whole peritoneum. It is possible in some instances to recover the meningococcus from the heart's blood when live culture has been used. The autolysate or killed culture may also kill. Even with live cultures death may be due to the poisonous bacterial protein injected rather than through its increase by multiplication. Rabbits injected either subcutaneously or intravenously may die.

Pathogenicity for Man.—The most marked lesions occur at the base of the brain. The cord is always affected. This is not true to the same extent in other bacterial infections. In some epidemics the course of the disease is very rapid. The mortality without serum treatment varies between 50 and 80 per cent.

Presence of Meningococci in the Nasal Cavity of the Sick and Those in Contact with Them.—In 6 of his 6 cases of meningitis Weichselbaum succeeded in obtaining diplococci from the nasal secretion. In 1901 Albrecht and Ghon demonstrated them in healthy individuals.

During the World War much work was done on the detection of meningococcus carriers and with the better culture media and technic of collecting material (use of West tube) large numbers of carriers were detected. Whether or not all of the strains isolated were pathogenic is a question.

Presence in other Localities.—In addition to the situation already noted, the meningococcus is frequently found in the blood in the early days of the disease. Elser found it in blood cultures in 10 out of 40

cases. In the severer cases the bacteriemia may be marked or even a septicemic picture may develop. The meningococci have also been found in the herpes and in the urine, a fact to be considered in ordering the hygiene of the sick room. Complications due to the meningococcus, otitis media, mastoiditis, irido-choroiditis, arthritis and endocarditis may occur. A pneumonia due to other bacteria may complicate a meningitis.

Meningococcus Vaccine.—Three injections, at weekly intervals, of 250,000,000, 500,000,000 and 1,000,000,000 respectively give rise to considerable antibody production. The vaccine has been used as a preventive during unusual prevalence of the disease. The statistics are too few to be conclusive. They seem to indicate that the procedure has value.

In treatment of cases Dubois and Neal have obtained favorable results with an autogenous vaccine when long-continued serum therapy has failed to cause complete recovery. The dosage range is from 100,000,000 to 1,000,000,000 or more, injections being given every two or three days.

Antimeningococcus Serum.—The intraspinal injection was introduced by Jochman. The value of the use of the serum by this route was definitely established by the investigations carried out under Flexner's direction. In his laboratory the serum has been used by the staff of the Meningitis Division and the mortality of the treated cases has ranged from 21 to 34 per cent. with an average of about 25 per cent.

A difficulty in the preparation of the antiserum is the varying prevalence of the meningococcus types. (See Agglutination Groups.)

In the attempt to make an antiserum to cover the more heterogeneous group one may obtain an antiserum which is not of sufficient potency for the more fixed groups. On the whole, the evidence indicates that an antiserum for the more fixed groups is the better compromise, as such an antiserum will (in the test-tube at least) show considerable group activity against the majority of meningococci. As was noted in Chapter XI every precaution should be taken to select dominant types and maintain them as such. (See Degradation under Agglutination.)

Administration of the Serum.—Dr. Neal finds that physicians tend to two extremes—an insufficient number of intraspinous injections or an excessive number with unnecessary amounts of serum. She recommends the following procedure: Perform a lumbar puncture and allow the fluid to drain into a sterile container[1] until the pressure is so reduced that only 3 to 4 drops come during a minute. The serum is then introduced through the needle by gravity, using a funnel and rubber tube. The average dose for adults is 20 to 40 c.c., for infants and children up to 20 c.c. The amount will depend on the amount of fluid withdrawn. The dose should usually be from 5 to 10 c.c. less.

In moderate or mild cases an injection every twenty-four hours will

[1] For laboratory examination. If the fluid is cloudy, the serum should be given; its continuance will depend on the laboratory findings.

suffice. More frequent injections are rarely indicated even in severe cases and not more than two each day are ever required. Injections should be continued until the fluid is shown to be sterile. If any sign of relapse develops the injections should be again started.

Usually 4 to 6 injections will suffice, but as many as 15 or more may be required. Due to the work of Herrick at army camps the idea has become very prevalent that intravenous injections should be generally employed. In civil life the markedly septicemic case is much less frequent than in camp epidemics. An initial intravenous injection may be given in marked septicemic cases. Intravenous injections are distinctly indicated where a persistent bacteriemia (as revealed by blood culture) or a persistent or recurring hemorrhagic rash occurs. As a rule, the blood concentration of antibodies will be kept up by the flow toward the blood of the antibodies introduced intraspinously. The serum injected intravenously will not appreciably influence the meningeal infection, as only a small percentage of the antibodies in the blood reaches the cerebrospinal fluids.[1]

Laboratory Diagnosis.—*Spinal Fluid.*—The fluid should be collected in a sterile container. It may be clear, cloudy, or bloody. If it is clear it may be a normal fluid or a fluid from a case of poliomyelitis or tuberculous meningitis. If the fluid is cloudy the infection may be due to the meningococcus, streptococcus, pneumococcus, influenza bacillus or other organisms. Very rarely one may obtain a cloudy fluid in a tuberculous case or a clear fluid from cases infected with the meningococcus, streptococcus, or other varieties noted above. The blood in a bloody fluid may be due to a previous hemorrhage or to the accidental piercing of a vein. The two conditions may be differentiated by centrifugalizing the fluid. If the supernatant fluid is yellow or reddish the hemorrhage is old. Clear fluid indicates a recent hemorrhage. Bloody fluids due to a hemorrhage rarely, if ever, coagulate on standing. Much blood in the fluid obscures the microscopic picture and unless the culture is positive it is difficult to make a diagnosis. The fluids to be examined should be centrifugalized. Clear fluids should always be centrifugalized preferably for one hour at high speed. The sediments should be used to make smears and cultures. The smears are examined: (1) For pus cells and (2) for tubercle bacilli if the fluid is clear or for other organisms if it is cloudy. The sediment from cloudy fluids are stained with Gram's stain. Tentative identification of the streptococcus, pneumococcus and influenza bacillus is aided by this method of staining. The finding of Gram-negative cocci either intra- or extracellular is presumptive evidence of meningococcic meningitis. As Gram-positive cocci will occasionally decolorize, it is desirable to verify by culture. Fluids should be examined at the earliest moment possible, for meningococci in fluids over twelve hours old are frequently so autolyzed that they will not grow.

[1] Neal: Abt's System of Pediatrics.

The following table gives the main differential points in making diagnosis from spinal fluids:

	Pressure.	Amount, c.c.	Appearance.	Cytology.	Bact.	Albumin.	Globulin.	Animal inoc.
Normal	Normal	5–10	Clear	Very few cells	Sterile	±	—	Negative
Meningismus	Increased	10–100	Clear	Very few cells	Sterile	±	—	Negative
Infantile paralysis	Increased	20–100	Clear; sometimes slight fibrin web	Early polynucleosis; later lymphocytosis up to 98 per cent; endothelial cells	Sterile	+–++	+++	Negative or pneumonia
Tuberculous meningitis	Increased	30–120	Clear; fibrin web	Lymphocytosis up to 98 per cent.	Tubercle bacilli	++–+++	++–+++	Tuberculosis in 4 weeks.
Epidemic cerebrospinal meningitis	Increased	5–120	Cloudy	Polynucleosis up to 98 per cent.	Meningococcus	++–++++	++–++++	
Meningitis due to other organisms	Increased	20–100	Cloudy	Polynucleosis up to 98 per cent.	Infecting organisms	++–++++	++–++++	

Other Gram-negative Cocci Resembling Meningococci.—Micrococcus Pharyngis (Siccus) (von Lingelsheim), Chromogenic Gram-negative Cocci, Micrococcus Catarrhalis.—These may be differentiated by cultural characteristics.

Differentiation with Sugars.—As a rule, the portion of the body from which the organisms are obtained reveals their identity. When this is insufficient cultural and serological tests are required.

The following table gives the chief sugar reactions of this group of microörganisms. (Elser and Huntoon.)

Organism.	Glucose.	Maltose.	Mannitose.	Levulose.	Saccharose.
Gonococcus	+	–	–	–	–
Meningococcus	+	+	–	–	–
M. Catarrhalis	–	–	–	–	–
M. Flavus	+	+	+	+	–
M. Pharyngis	+	+	+	+	+

Serological tests may also be employed.

Other Organisms Exciting Meningitis.—1. The tubercle bacillus is the most frequent cause of meningitis due to other bacteria than the meningococcus.

2. The pneumococcus is one of the most frequent exciters of meningitis, both as a primary and a secondary infection.

3. The Streptococcus pyogenes and the staphylococcus cause meningitis, usually secondary to some other infection, such as otitis, tonsillitis, erysipelas, endocarditis, suppurating wound of scalp and skull, etc.

4. The Bacillus influenzæ is a relatively frequent cause of meningitis. Infection is often apparently primary, but may be secondary to infection of the lungs, bronchi and the nasal cavities and accessory sinuses. (See chapter on Influenza Bacillus.)

5. The colon bacillus, the typhoid bacillus, the plague bacillus and the glanders bacillus may cause a complicating purulent meningitis.

6. In isolated cases of meningitis complicating otitis media and other infections, other microörganisms, such as the Micrococcus catarrhalis,[1] Micrococcus tetragenus, the Bacillus pyocyaneus, the gonococcus, actinomyces,[2] yeasts,[3] etc., may be found.

Meningitis due to other organisms than the meningococcus is almost invariably fatal.

The following table was compiled by Neal[4] of our laboratory from cases of meningitis occurring in our service.

DISTRIBUTION OF MENINGITIS ACCORDING TO AGE AND ETIOLOGY.

Age.	Tubercle bacillus.	Meningococcus.	Pneumococcus.	Streptococcus.	Influenza bacillus.	Staphylococcus.	Colon bacillus.	Total.
3 months	5	24	3	11	2	0	3	48
3 to 6 months	21	58	4	6	8	0	0	97
6 to 12 "	78	68	11	3	14	2	0	176
Total No. cases under 1 year	104	149	18	19	24	2	3	321
1 to 2 years	178	79	8	9	14	2	1	291
2 to 3 "	80	36	9	6	6	1	0	138
3 to 5 "	99	80	6	11	1	2	0	199
5 to 10 "	96	105	16	20	3	0	1	241
10 to 20 "	57	101	7	5	3	2	0	175
Over 20 "	48	74	22	12	0	2	0	158
Totals	662	625	86	83	51	11	5	1523

MICROCOCCUS (NEISSERIA) CATARRHALIS.

Micrococci somewhat resembling meningococci are found in the mucous membranes of the respiratory tract. At times they cause catarrhal inflammation of the mucous membranes and pneumonia. These cocci are at present included under the designation of Micrococcus catarrhalis.

Microscopic Appearance.—They usually occur in pairs, sometimes in fours; never in chains. The cocci are coffee-bean in shape, slightly larger than the gonococcus, are negative to Gram's stain, non-motile and do not produce spores.

Cultivation.—They grow between 20° and 40° C., best at 37° C. and less rapidly at somewhat lower temperatures, developing on ordinary nutrient agar as grayish-white or yellowish-white, circular colonies of the size of meningococci. The borders of the colonies are irregular and abrupt as though gouged out. The colonies have a mortar-like consistency. On serum-agar media the growth is more luxuriant. Gelatin is not liquefied. Bouillon is clouded, often with the development of a pellicle. Milk is not coagulated, but dextrose serum media may be. Gas is not produced. (See Table p. 364 for sugar reactions.)

[1] Garland: Am. Jour. Dis. Child., 1923, **26**, 600 (also quoting Neal's case.)
[2] Shippiro and Neal: Jour. Am. Med. Assn., 1923, **81**, 212.
[3] Elmendorf and Neal: Arch. Ped., 1924.
[4] Jour. Am. Med. Assn., 1924.

Location of Organisms.—In the secretion of normal mucous membranes they are occasionally present. In certain diseased conditions of the mucous membranes they may be abundant.

Pathogenic Effects in Animals.—For white mice, guinea-pigs, and rabbits, some cultures are as pathogenic as meningococci, while others are less so.

Differential Points Distinguishing them from the Meningococci.—These organisms have undoubtedly been at times confused. Some assert that the meningococci grow only above 25° C. Many cultures of meningococci grow below this point. Some assert that the meningococci will not grow on 5 per cent. glycerin agar. Many undoubted cultures do. The probability is that the organisms described by different writers as Micrococcus catarrhalis were not at all the same variety, and some of them may have been meningococci.

CHAPTER XVIII.

THE GONOCOCCUS OR NEISSERIA GONORRHEÆ.

The period at which gonorrhea began to afflict man is unknown. The earliest records make mention of it. Wherever civilized man has penetrated, gonorrhea is prevalent among the people. Except for a period after the fifteenth century it was generally recognized as a communicable disease and laws were made to control its spread. The differentiation between the lighter forms of gonorrhea and some other inflammations of the mucous membranes was, however, almost impossible until the discovery of the specific microörganism by Neisser in 1879.

The organism was first observed in gonorrheal discharges and described by him under the name of "gonococcus;" but though several attempted to discover a medium upon which it might be cultivated, it was reserved for Bumm, in 1885, to obtain it in pure culture upon coagulated human blood serum, and then after cutivating it for many generations to prove its infective virulence by inoculation into man. The researches of Neisser and Bumm established beyond doubt that this organism is the specific cause of gonorrhea in man. In adults gonorrhea of the genital tract is in almost all cases transmitted through sexual intercourse. Gonorrheal ophthalmia is a frequent accidental infection at birth, and vaginitis in the young child is frequently produced by the carelessness of the nurse or mother carrying infection.

Microscopic Appearance.—These micrococci occur mostly in the form of diplococci. The bodies of the diplococci are broadened and in stained preparations show an unstained division or interspace between two flattened surfaces facing one another, which give them their characteristic "coffee-bean" or "kidney" shape. The older cocci lengthen, then become constricted in their middle portion, and finally divide, making new pairs (Fig. 109). The diameter of an associated pair of cells varies according to their stage of development from 0.8μ to 1.6μ in the long diameter—average about 1.25μ—by 0.6μ to 1μ in the cross diameter.

Extracellular and Intracellular Position of Gonococci.—In gonorrhea, during the earliest stages before the discharge becomes purulent, the gonococci are found mostly free in the serum or plastered upon the epithelial cells, but later almost entirely in smaller or larger groups of *spaced* diplococci in or upon the pus cells and epithelial cells, and always extranuclear. The spaced appearance makes them look as if they were surrounded by a capsule (Isreali[1]). With the disappear-

[1] Jour. Am. Med. Assn., 1921, **76**, 1497.

ance of the pus formation more free gonococci appear. Discharge expressed from the urethra usually contains more free organisms than the natural flow. Gonococci sometimes appear irregular or granular, the so-called involution forms. These are found particularly in older cultures and in chronic urethritis of long standing. Single pus cells sometimes contain as many as one hundred gonococci and seem to be almost bursting and yet show but slight signs of injury. These diplococci are also found in or upon desquamated epithelial cells (Fig. 110). In gonorrhea of the conjunctiva the gonococci are contained in the epithelial cells, sometimes in large numbers. They form dense groups which contain forms similar to those seen in older cultures, showing metachromatic granules in round, swollen, pale blue bodies. These groups finally present an appearance somewhat like the cell inclusions found in "trachoma" (p. 476).

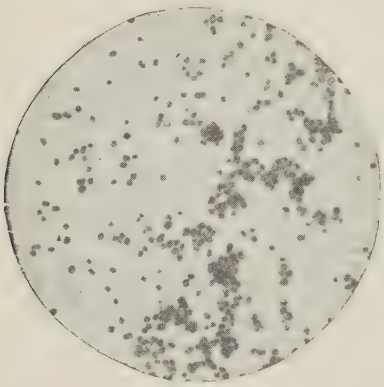


Fig. 109.—Smear from pure culture of gonococcus on agar. × 1100 diameters. (Heiman.)

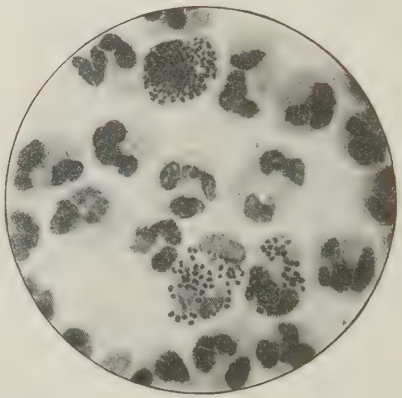


Fig. 110.—Gonococcus within pus cells, fuchsin stain. ×1000 diameters. (Fränkel and Pfeiffer.)

Staining.—The gonococcus *stains* readily with the basic anilin colors (see Stains). Löffler's solution of methylene blue is one of the best staining agents for demonstrating its presence in pus, for, while staining the gonococci deeply, it leaves the cell protoplasm but faintly stained. Fuchsin is apt to overstain the cell substance. Beautiful double-stained preparations may be made from gonorrheal pus by treating glass slide spreads with methylene blue and eosin. Numerous methods for double staining have been employed, with the object of making a few gonococci more conspicuous. None of them has any specific characteristics such as the Gram stain. It is now established that gonococci from fresh cultures and from recent gonorrheal infections, when properly treated by Gram's method, are quickly and surely robbed of their color and take on the contrast stains. The removal of the stain from gonococci in old flakes and threads from chronic cases is not so certain. This difference is mostly due to the fact that equally uniform specimens cannot be

prepared. This method of staining cannot be depended upon alone absolutely to distinguish the gonococcus from all other diplococci found in the urethra and vulvo-vaginal tract, for, especially in the female, other diplococci are occasionally found which are also not stained by Gram's method. It serves, however, to distinguish this micrococcus from the common pyogenic cocci, which retain their color when treated in the same way, and in the male urethra it is practically determinative, as few organisms have been found in that location which in morphology and staining are identical with the gonococcus. It is certainly the most distinctive characteristic of the staining properties of the gonococcus, and it is a test that should never be neglected in differentiating this organism from others which are morphologically similar.

Biology.—Grows best at blood temperature; the limits being roughly 25° and 40° C. It is a facultative anaërobe. It is not motile and produces no spores.

Culture Media.—The gonococcus requires for its best growth the addition to nutrient agar of a small percentage of blood serum or some equivalent. (See Media.) We have found that they grow very well on heated blood mixtures, such as vitamin blood agar. Ruediger[1] also obtained good results with similar media with partial exclusion of air, thus confirming our observations on the facultative anaërobic nature of this organism.

After continued cultivation gonococcus cultures frequently grow on media containing no serum. Occasional strains grow on ordinary glycerin or glucose nutrient agar and even on plain nutrient agar from the start, but their growth is more capricious in such media.

Viability.—Cultures usually die in forty-eight to seventy-two hours when kept at room temperature. In the ice-box they may live for several weeks. They frequently live for one week in the thermostat at 36° C. on and in semisolid media.

Appearance of Colonies.—A delicate growth is characteristic. At the end of twenty-four hours there will have developed translucent, very finely granular colonies, with an occasional indentation or scallop of the margin. The margin is sometimes scarcely to be differentiated from the culture medium. In color they are grayish-white, with a tinge of yellow. The texture is finely granular at the periphery, presenting yellowish punctated spots of higher refraction in and around the center (Fig. 111).

Surface Streak Culture.—Translucent grayish-white growth, with rather thick edges.

Resistance.—The gonococcus has but little resistance toward outside influences. It is killed by weak disinfecting solutions and by desiccation in thin layers. In comparatively thick layers, however, as when gonorrheal pus is smeared on linen, it has lived for forty-nine days, and dried on glass, for twenty-nine days (Heiman). It is killed at a temperature of 60° C. in less than ten minutes.

[1] Jour. Infect. Dis., 1919, **24**, 376.

Pathogenesis.—They are non-transmissible as gonorrhea to any test animal. But both the living and dead gonococci contain toxic substances. Injected in considerable amounts into rabbits, they cause infiltration and often necrosis. Applied to the urethral mucous membrane of human beings there is produced an inflammation of short duration. In gonorrhea the secretion is believed to be due to intracellular toxins. Repeated injections give only slight immunity. The filtrate of recent gonococcus cultures contains little toxic substance.

The etiological relation of the gonococcus to human gonorrhea has been demonstrated beyond question by the infection of a number of healthy men with the disease by the inoculations of pure cultures of the microörganism.

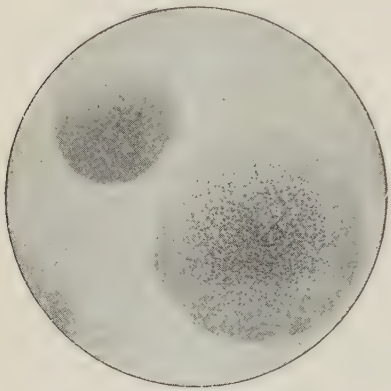


Fig. 111.—Colonies of gonococci on pleuritic fluid agar. (Heimann.)

Disease Conditions Excited by Gonococci.—Infections due to this organism are usually restricted to the mucous membranes of the urethra, prostate, neck of bladder, cervix uteri, vagina, and conjunctiva. The conjunctival, vaginal, and rectal mucous membranes are much more sensitive in early childhood than in later life. The usual course of the inflammation is as follows: The gonococci first increase upon the mucous membranes which show congestion, infiltration with serous exudate and accumulation of leukocytes. The cocci then penetrate the epithelial layer down to the submucous connective tissue. The infection is followed by recovery or a chronic inflammation. The original infection of the urethra or vagina and cervix may remain localized or spread to adjacent parts or through blood and lymph be carried to all parts of the body. Gonococci thus cause some cases of endometritis, metritis; salpingitis, oöphoritis, peritonitis, prostatitis, cystitis, epididymitis, and arthritis. Abscesses of considerable size, periostitis, and otitis are occasionally due to the gonococcus. Gonococcus endocarditis and septicemia are not infrequent. The latter may develop without demonstrable local lesions.

Ophthalmia Neonatorum.—We have corroborated the statement of Stephenson and others that the gonococcus is not the only cause of ophthalmia neonatorum; in fact, in only about two-thirds of these cases is the gonococcus the cause of the inflammation.[1]

Complications.—General infections with gonococci are often followed or accompanied by neuralgic affections, muscle atrophies, and neuritis. Urticaria occasionally occurs.

Immunity.—Immunity in man after recovery from infection seems to be only slight in amount and for a short period if present at all. It is known that the urethra in man or cervix uteri in woman may contain

[1] Williams and Rosenberg: Arch. of Ophthal., 1916, **45**, 109.

gonococci which lie dormant and may be innocuous in that person for years, but which may at any time excite an acute gonorrhea in another individual or, under stimulating conditions, in the one carrying the infection. Animals, may, however, be immunized, and their blood appears to be bactericidal.

Gonococcus Vaccine.—Urethritis is apparently uninfluenced by the use of vaccines nor is the number of complications appreciably reduced. Vaginitis in children is little influenced. There are some who believe that epididymitis and prostatitis are beneficially influenced. Pelvic infection and general infection are not benefited. Periarthritis, however, seems to be definitely benefited. The dose for subcutaneous injection is from 25,000,000 to 500,000,000. A moderate reaction seems to be followed by more definite therapeutic effect. Based on this, Bruch and Summers advocated the intravenous injection. This method is apparently more efficacious. Further experience indicates that the results are due to non-specific protein effect. Similar curative action is obtained with typhoid vaccine or even with sterile milk.

Antigonococcus Serum.—In adequate doses, 50 c.c. or more, given intravenously, there is an apparent benefit in septicemic cases. Injections of the serum have also been recommended to prevent complicating arthritis, the injections to be given at the first indication of joint involvement. It seems that the usefulness of the antiserum is limited to the acute and generalized infections.

Complement Fixation.—By the method of McNeil (Chapter XII) very good results have been obtained in the deep-seated chronic infections, but the reverse is true in the superficial acute cases. The method is used regularly in the New York City Health Department.

Agglutination.—Torrey's[1] second series of studies on the absorption of agglutinins of gonococci isolated from cases of acute and chronic gonorrhea led him to the conclusion that there are *not* clear-cut agglutinative types, that there seems to be a marked tendency to instability of antigen constitution, but there are certain strains that are highly generalized and so representative of a large part of the gonococcus group. Strains from children are no different from strains from men. A few representative strains with generalized relationships have been designated for use in preparing antigens and antiserums.

Duration of Infections and of Contagious Period.—There is no limit to the time during which a man or woman may remain infected with gonococci and infect others. We had one man under observation in which twenty years had elapsed since exposure to infection, and yet the gonococci were still abundant. It is now well established that most of the inflammations of the female genital tract are due to gonococci and that many of such infections are produced in innocent women by their husbands who are suffering from latent gonorrhea.

Bacteriological Identification of the Gonococcus.—In view of the fact that occasional non-gonorrheal forms of urethritis exist, and also that micrococci morphologically similar to Neisser's diplococcus are at times found in the normal vulvo-vaginal tract of adults, it becomes a

[1] Jour. Immunol., 1922, 7, 305.

matter of importance to be able to detect gonococci when present, and to differentiate these from the non-specific organisms. Besides this, the gonococci which occur in old cultures and in chronic urethritis of long standing sometimes take on a very diversified appearance. From a medico-legal and social standpoint, therefore, the differential diagnosis of the gonococcus has in certain cases a very practical significance.

There are three methods of differential identification now available—the microscopic, the cultural and complement fixation. The method employed in the last test is given in Chapter XII. Animal inoculations are of no value, as animals are not susceptible, and, of course, human inoculations are usually impossible. In the microscopic diagnosis it should be borne in mind that after the acute serous stage has passed, the specific gonococci in *carefully made* preparations are always found largely within the pus cells. Diplococci morphologically similar to gonococci occurring in other portions of the field and outside of the pus cells should not be considered specific by this test only. It should also be remembered that the gonococci are decolorized by Gram's method, while other similar micrococci which occur in the urethra are, as a rule at least, not so decolorized. Organisms having these characteristics can for all practical purposes be considered as certainly gonococci if obtained from the urethra. From the vulvo-vaginal tract the certainty is not so great, since other diplococci are found in pus from this area more frequently than from the urethra, which stain as gonococci; here cultures should also be made.

In *chronic urethritis* Heimann allows the patient to void his urine either immediately into two sterilized centrifuge tubes or first into two sterile bottles. The first tube will contain threads of the anterior urethra; the second tube will be likely to contain secretion from the posterior urethra and from the prostate gland if, while urinating, the patient's prostate be pressed upon with the finger. Tubes containing such urine are placed in the centrifuge and whirled for three minutes at 1200 or more revolutions per minute, at which speed the threads are thrown down. The centrifugalized sediment will be found to contain most of the bacteria present, epithelial cells, and, at times, spermatozoa.

When the examinations are negative and t is important to be certain either massage or injections of a solution of silver nitrate may be employed. The latter by causing a temporary irritation with increase of secretion will almost surely cause a discharge of gonococci if any infection is present. It has been recommended that inoculations of vaccine be given to provoke an increase of the number of gonococci in chronic cases. Pearson[1] states that this treatment gave positive results in 96 out of 100 consecutive cases.

In acute cases where the pus is abundant the specimen for examination may be collected by passing a sterilized platinum-wire loop as far up into the urethra as possible and withdrawing some of the secretion.

In vulvo-vaginitis the procedure should be as follows:

[1] Jour. of Urology, 1918, **2**, 455.

To obtain the vaginal material the labia are held well apart by an attendant wearing sterile rubber gloves. A sterile slender cotton swab is used which passes easily into the vagina without touching any external part but entrance. (If pus from the cervix is desired a speculum should be used.) The swab is rubbed gently about vaginal wall, then withdrawn and rolled (not rubbed) quickly over a slide (slide sterilized and held face down if culture is to be made). In making this spread care is used not to pass the swab over the same surface twice. In this way a beautifully spread film is made. The swab is then returned to its holder (and covered and numbered if culture is to be made).

The technic for making culture is as follows: A small amount of rich sterile ascitic fluid is added to the tubes containing the swab. (If patient is at a distance from laboratory the ascitic fluid is sent in a separate tube, and is inoculated from swab just after smear is made; then swab is withdrawn from ascitic fluid tube, placed in its own tube, and both tubes are sent immediately to the laboratory.) After being stirred up in this fluid the swab is withdrawn and smeared in streaks radiating from the centre over ascitic agar (1–4) plate containing 2 per cent. glucose. Other plates of the same medium are streaked with platinum loopfuls of the ascitic fluid emulsion. From 2 to 4 plates are made and placed in thermostat at 36° C. After twenty-four hours these are examined and if gonococcus-like colonies are seen they are fished. Then a smear is made from the whole of one of the most characteristic streaks, stained by Gram's method and examined for gonococcus-like organisms.

Grouping Cases.—From the microscopic examination of well-made and well-stained smears (stained by Gram's method), and, when necessary, from cultures and from clinical appearance as well, the cases are divided into four groups, as follows:

1. *Positive cases, i. e.*, those showing leukocytes filled with morphologically typical gonococci in smear or showing typical cultures, or showing both.
2. *Suspicious cases, i. e.*, those showing in smears any suspicious intracellular diplococci and 50 per cent. or more of polymorphonuclear leukocytes.
3. *Observation cases, i. e.*, those showing in smears 50 per cent. or over of polymorphonuclear leukocytes, but no suspicious intracellular diplococci; or those having the clinical symptoms of discharge and inflammation and showing less than 50 per cent. of polymorphonuclear leukocytes.
4. *Negative cases. i. e.*, those showing in smears less than 50 per cent. polymorphonuclears, and no suspicious intracellular diplococci, and no clinical evidence of the disease.

Isolation of Groups.—Each group is kept isolated.

Later Smears.—From the first three groups smears are made once a week until a negative smear is obtained. From negative groups smears are made if any suspicious symptoms appear.

Negative Diagnosis from Later Smears.—Three successive well-made and well-stained negative smears from first three groups, at intervals of not longer than three days, are considered a negative diagnosis.

Later Cultures.—From cases where morphologically typical gonococci persist in smear, cultures may be made and gonococcus-like organisms isolated and studied to find out if they are true gonococci.

Bacteria Resembling Gonococci.—A few micrococci which resemble gonococci in form and staining have been described. These assume importance largely because they may be confused with the gonococcus. They occur occasionally on the conjunctival and vaginal mucous membranes. One of these microörganisms, the *Micrococcus catarrhalis* (see p. 365), has an importance of its own. Others are probably unimportant. When absolute certainty is demanded cultural and serological tests must be applied. The method of differentiation from meningococci is given on p. 364.

CHAPTER XIX.

THE BACILLUS AND THE BACTERIOLOGY OF DIPHTHERIA.

The lesions of diphtheria are caused by an extracellular toxin produced by the diphtheria bacillus. The endotoxin is of minor importance. The poison elaborated at the seat of the exudate causes intense local inflammation, while in the more severe cases the absorbed poison diffused throughout the body causes widespread cellular injury.

Historical Notes.—This specific contagious disease can be traced back under various names to almost the Homeric period of Grecian history.

In 1771 Bard, an American, advanced strong reasons for believing that membranous croup and pharyngeal diphtheria were different manifestations of the same disease process.

In 1821 Bretonneau published his first essay on diphtheria in Paris and gave to the disease its present name. His observations were so extensive and so correct that little advance in knowledge took place until the causal relations of the diphtheria bacilli and their associated microörganisms to the disease began to be recognized.

Evidence of Causal Relationship.—In the year 1883 bacilli which were very peculiar and striking in appearance were shown by Klebs to be of constant occurrence in the pseudomembranes from the throats of those having the characteristic symptoms of diphtheria. One year later Löffler separated these bacilli from the other bacteria present in the exudate and grew them in pure culture. They have, therefore, frequently been called Klebs-Löffler bacilli. When Löffler simply placed the bacilli upon the mucous membranes of animals no infection resulted, but when he inoculated the bacilli upon the abraded mucous membrane of susceptible animals more or less characteristic pseudomembranes were produced, and often death or paralysis followed with characteristic lesions. These animal experiments have been fortified by a number of accidental human inoculations in laboratories with pure cultures of bacilli with subsequent development of characteristic diphtheria. Since then we have learned that besides the diphtheria bacilli there are diphtheria-like bacilli, which closely resemble the diphtheria bacilli but produce no diphtheria toxin, and another group of bacilli called diphtheroids, which differ from the diphtheria bacilli not only in not producing the extracellular toxin, but also in their fermentative reactions on carbohydrates.

THE DIPHTHERIA BACILLUS (CORYNEBACTERIUM DIPHTHERIÆ).

Morphology.—When hanging drops of diphtheria exudates or cultures grown on blood serum for ten hours or more are examined, the diph-

theria bacilli are found to possess the following morphological characteristics: The diameter of the bacilli varies from 0.3μ to 0.8μ and the length from 1μ to 6μ. They occur singly and in pairs (see Figs. 112 to 119) and very infrequently in chains of three or four. The rods are straight or slightly curved, and usually are not uniformly cylindrical throughout their entire length, but are swollen at the end, or pointed at the ends and swollen in the middle portion. The average length of the bacilli in pure cultures from different sources frequently varies greatly, and even from the same culture individual bacilli differ much in their size and shape. This is especially true when the bacilli are grown in association with other bacteria or are allowed to grow for longer periods than twelve hours. The two bacilli of a pair may lie with their long diameter in the same axis or at an obtuse or an acute angle. The bacilli possess no spores, but have in them highly refractive bodies, some of which are the starting point for new bacilli. There are no flagella. For mode of division see p. 36.

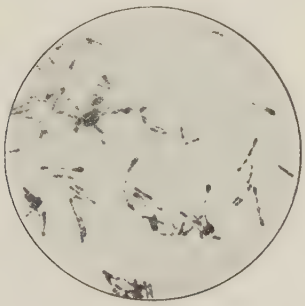


FIG. 112.—One of the very characteristic forms of diphtheria bacilli from blood-serum cultures, showing clubbed ends and irregular stain. × 1100 diameters. Stain, methylene blue.

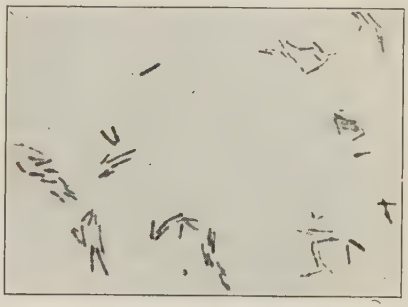


FIG. 113.—Diphtheria bacilli characteristic in shapes, but showing even staining. × 1000 diameters. Stain, methylene blue.

Staining.—Diphtheria bacilli *stain* readily with ordinary anilin dyes, and retain fairly well their color after staining by Gram's method. With Löffler's alkaline solution of methylene blue the bacilli from blood-serum cultures especially, and from other media less constantly, stain in an irregular and extremely characteristic way. (See Fig. 112.) The bacilli do not stain uniformly. In many cultures round or oval bodies, situated near the ends or in the central portions, stain much more intensely than the rest of the bacillus, usually showing metachromatism (metachromatic granules. See page 80 and Plate II). Sometimes these highly stained bodies are thicker than the rest of the bacillus; again, they are thinner and surrounded by a more slightly stained portion. Other bacilli have barred staining. The bacilli stain in this peculiar manner at a certain period of their growth, so that only a portion of the organisms taken from a culture at any one time will show the characteristic staining. The young cultures have the most regular forms,

an eighteen-hour growth showing more clubbed forms than at twelve hours. After twenty-four hours the majority of the bacilli do not stain

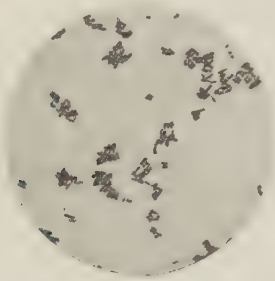


FIG. 114.—B. diphtheria agar culture. Bacilli small and uniform in shape. × 1000 diameters.

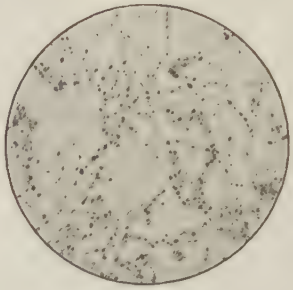


FIG. 115.—Diphtheria-like bacilli, showing stain with Neisser's solutions, but producing no toxin. This appearance was formerly supposed to be characteristic of virulent bacilli. Bodies of bacilli in smear, yellowish-brown; granules dark blue.

FIG. 116.—Extremely long form of diphtheria bacillus. This culture has grown on artificial media for thirty-five years and produces very potent toxin. × 1100 diameters.

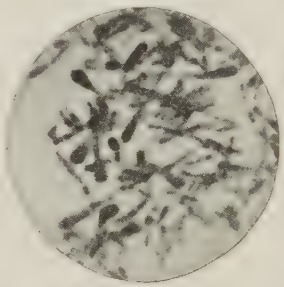


FIG. 117.—B. diphtheriæ. Forty-eight hours' agar culture. Thick, Indian-clubbed rods and moderate number of segments. One year on artificial culture media. × 1410 diameters.

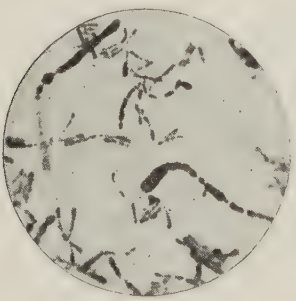


FIG. 118.—B. diphtheriæ. Forty-eight hours' agar culture. Many segments; long Indian-clubbed ends. One year on artificial media. × 1410 diameters.

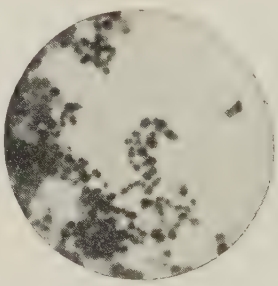


FIG. 119.—B. diphtheriæ. Twenty-four hours' agar culture. Unusual coccus forms. Segmented granular forms on Löffler's serum. Only variety found; in cases of diphtheria at a children's home. × 1410 diameters.

quite as well, but many show characteristic forms. In still older cultures it is often difficult to stain the bacilli, and the staining, when it does occur, is frequently not at all characteristic. The same round or oval bodies which take the methylene blue more intensely than the remainder of the bacillus are brought out still more distinctly by the Neisser stain (see under Staining Methods).

The Neisser stain and many others have been advocated in order to separate the virulent from the non-virulent bacilli, without the delay of inoculating animals; but in our hands, with a very large experience, neither the Neisser stain nor other stains have given any more information as to the virulence of the bacilli than the well-ripened good staining methylene-blue solution of Löffler. A small proportion of strains of virulent bacilli fail to show a marked characteristic stain, and quite a few diphtheroids show the dark bodies. Then there are those bacilli which seem to have all the staining and cultural characteristics of the virulent bacilli, and yet produce no diphtheria toxin. As will be stated more fully later, animal inoculations with the suspected bacilli together with control injections of diphtheria antitoxin constitute the only practical method for separating harmless bacilli from those capable of producing diphtheria.

The Morphology of the Diphtheria Bacillus on Serum-free Media.—This varies considerably with different culture media employed. On glycerin agar or simple nutrient agar there are two distinct types. One grows as smaller and, as a rule, more regular forms than when grown on serum culture media (Fig. 114). The other type shows many thick, Indian-club forms with a moderate number of segments (Figs. 117 and 118). Short, spindle-, lancet-, or club-shaped forms, staining uniformly, are all observed.

Biology.—The Klebs-Löffler bacillus is non-motile and non-liquefying. It is *aërobic* and facultative *anaërobic*. It grows most readily in the presence of oxygen. It does not form spores. It begins to develop but grows slowly at a temperature of 20° C. or even less. It attains its maximum development at 37° C. In old cultures in fluid media Williams has observed fusion of one bacillus with another. The fused forms live the longest.

Growth on Culture Media.—Blood Serum.—Blood serum, especially coagulated in the form of Löffler's mixture, is a favorable medium for the growth of the diphtheria bacillus, and is used particularly for diagnostic purposes in examining cultures from the throats of persons suspected of having diphtheria. Cultures grown on coagulated horse serum may be equally characteristic if a vitamin broth base is used. For its preparation see Chapter on Media. If we examine the growth of diphtheria bacilli in pure culture on blood serum, we shall find at the end of from eight to twelve hours small colonies, which appear as pearl-gray, whitish-gray or, more rarely, yellowish-gray, slightly raised points. The colonies when separated from each other may increase in forty-eight hours so that the diameter may be one-eighth of an inch. The colonies lying together become confluent and fuse into one mass when the serum

is moist. The diphtheria colonies after a growth of twelve hours become larger than those of the streptococci but remain smaller than those of the staphylococci.

Growth on Agar.—On 1 per cent. slightly alkaline, nutrient or glycerin-agar the growth of the diphtheria bacillus is less certain and luxuriant than upon blood serum; but the appearance of the colonies when examined under a low-power lens, though very variable, is often far more characteristic (Fig. 120). For this reason nutrient agar in Petri dishes is used to obtain diphtheria bacilli in pure culture. While certain strains grow well from the start on nutrient agar, other strains do not. Some of these, after having been transplanted for several generations on serum culture media, grow well, or fairly well, on suitable nutrient agar, but when fresh from pseudomembranes they grow on this medium with great difficulty, and the colonies develop so slowly as to be covered up by the more luxuriant growth of other bacteria when present; or they may fail to develop at all.

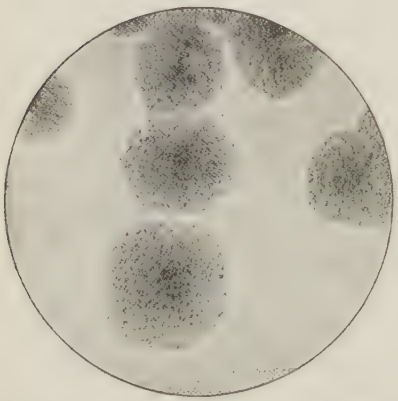


FIG. 120.—Colonies of diphtheria bacilli. × 200 diameters.

If the colonies develop deep in the substance of the agar they are usually round or oval, and, as a rule, present no extensions; but if near the surface, commonly form one. When colonies develop entirely on the surface they are more or less coarsely granular, and usually have a dark center and vary markedly in their thickness. The colonies from some are almost translucent; from others, are thick and almost as luxuriant as the staphylococcus. The edges are sometimes jagged, and frequently shade off into a delicate lace-like extension; at other times the margins are more even and the colonies are nearly circular. When grown for some time on nutrient media some of the bacilli become less virulent. When plate cultures are made from such bacilli the surface colonies which develop are apt to grow in a more compact manner than the spreading growth of the fully virulent bacilli. The two types of colonies correspond to rough and smooth colonies of some varieties of bacteria.

Peculiarities in the growth of the diphtheria bacillus upon agar are of practical importance. If a large number of the bacilli from a recent culture are implanted upon a properly prepared agar plate, a certain and fairly vigorous growth will always take place. If, however, the agar is inoculated with an exudate from the throat, which contains but a few bacilli, no growth whatever may occur, while the tubes of coagulated blood serum inoculated with the same exudate contain the bacilli abundantly. Because of the uncertainty, therefore, of obtaining a growth by the inoculation of agar with bacilli unaccustomed to this medium, agar is not a reliable medium for use in primary cultures for diagnostic purposes. A mixture composed of 2 parts of a 1.5 per cent. nutrient

agar and 1 part of sterile ascitic fluid makes a medium upon which the bacillus grows much more luxuriantly, but not so characteristically.

Growth in Bouillon.—The diphtheria bacilli from about one-half the freshly isolated cultures grow readily in broth slightly alkaline to litmus; the other strains grow feebly. The characteristic growth in neutral bouillon is one showing fine grains. These deposit along the sides and bottom of the tube, leaving the broth nearly clear. A few cultures in neutral bouillon and many in alkaline bouillon produce for twenty-four or forty-eight hours a more or less diffuse cloudiness, and frequently a film forms over the surface of the broth. On shaking the tube this film breaks up and slowly sinks to the bottom. This film is apt to develop during the growth of cultures which have long been cultivated in bouillon and, indeed, after a time the entire development may appear on the surface in the form of a friable pellicle. The diphtheria bacillus in its growth, causes a fermentation of meat sugars and glucose, and this, if these are present, changes the reaction of the bouillon, rendering it distinctly less alkaline within forty-eight hours, and then, after a variable time, when all the fermentable sugars have been decomposed, more alkaline again through the progressing fermentation of other substances. Among the products formed by its growth is the diphtheria toxin.

Growth in Ascitic or Serum Bouillon.—All varieties of diphtheria bacilli grow well in this medium, even when first removed from the throat. They almost always form a slight pellicle at the end of twenty-four hours which increases by the end of forty-eight hours. This culture medium is, as first pointed out by Williams, of the greatest value in attempts to get pure cultures of the diphtheria bacillus from solidified serum cultures containing few bacilli among many other bacteria. Plate cultures are made from the pellicle. The fluid is prepared by adding to the nutrient bouillon 25 per cent. ascitic fluid or blood serum.

Growth in Gelatin.—The growth on this medium is much slower, more scanty, and less characteristic than that on the other media mentioned. This largely is on account of the lower temperature at which it must be used.

Growth in Milk.—The diphtheria bacillus grows readily in milk, beginning to develop at a comparatively low temperature (20° C.). The milk remains unchanged in appearance, as the lactose is not fermented.

Pathogenesis.—In Lower Animals.—The diphtheria bacillus, through its toxins is, when injected into their bodies, pathogenic for guinea-pigs, rabbits, chickens and cats; also for dogs, goats, cattle and horses, but hardly at all for rats and mice. In spite of its pathogenic qualities for these animals, true diphtheria occurs in them with extreme rarity. As a rule, supposed diphtheritic inflammations in them are due to other bacteria which cannot produce disease in man. The cat is the only animal that we have known to contract true diphtheria from contact with the disease. Cobbett reports a case in a colt. Cows with pustules on the teats have become infected. At the autopsy of animals dying from the poisons produced by the bacilli, the characteristic lesions described by Löffler are found. At the seat of inoculation there is a grayish focus surrounded by an area of congestion; the subcutaneous tissues for some distance around are edematous; the adjacent lymph nodes are swollen; and the serous cavities, especially the pleura and the pericardium, frequently contain an excess of fluid usually clear, but at times turbid; the lungs are usually congested, the suprarenals are

markedly congested. In the organs are found numerous smaller and larger masses of necrotic cells, which are permeated by leukocytes. The heart and certain voluntary muscular fibers and nervous tissues usually show degenerative changes. From the area surrounding the point of inoculation virulent bacilli may be obtained, but in the internal organs they are only occasionally found, unless enormous numbers of bacilli have been injected. Paralysis, commencing usually in the posterior extremities, and then gradually extending to the whole body, and causing death by paralysis of the heart or respiration, is also produced in many cases in which the inoculated animals do not succumb to a too rapid intoxication. In a number of animals we have seen recovery take place three to six weeks after the onset of the paralysis.

Tissue Changes in Natural (Human) Infection.—The characteristic lesions are a pseudomembranous inflammation on some of the mucous membranes, or occasionally on the surface of wounds, and the general hyperplasias and parenchymatous inflammations produced by the absorbed toxic substances. Pneumonia is apt to occur as a complication of laryngeal diphtheria. The membrane may be simply a thin pellicle, which is easily removed without causing bleeding, or it may be thick and firmly attached and leaving when removed a ragged bleeding surface. The tissue beneath the pseudomembrane is always intensely congested and often hemorrhagic. The cells show marked degenerative changes.

Diphtheria Toxin.—This poison was assumed by Löffler (1884) to be produced by the bacilli, but it was first separated from them by Roux and Yersin, who obtained it from broth cultures of the living bacilli by filtration through porous porcelain.

The Production of Toxin in Culture Media.—The artificial production of toxin from cultures of the diphtheria bacillus has been found to depend upon definite conditions, which are of practical importance in obtaining toxin for the Schick test, for toxin-antitoxin and for the inoculation of horses. The researches of Roux and Yersin laid the foundation of our knowledge. Their investigations have been continued by Theobald Smith, Spronck, ourselves, and others. After an extensive series of investigations we (Park and Williams) came to the following conclusions: Toxin is produced by virulent diphtheria bacilli at all times during their life when the conditions are favorable. Diphtheria bacilli may find conditions suitable for luxuriant growth, but unsuitable for the production of toxin. The requisite conditions for good development of toxin, as judged by the behavior of a number of cultures, are a temperature of from about 32° to 35° C., a suitable culture medium, such as a 2 per cent. peptone nutrient bouillon made from fresh veal, of an alkalinity which should be about 9 c.c. of normal soda solution per liter above the neutral points of litmus, and prepared from a suitable peptone and meat. The culture fluid should be in comparatively thin layers and in large-necked Erlenmeyer flasks, so as to allow of a free access of air. The greatest accumulation of toxin in bouillon is after a duration of growth of the culture of from five to nine days, according to the peculiarities of the culture used and methods employed. In our experience the variations in the small amount of muscle sugar present in the meat makes no appreciable difference in the toxin produced when a vigorously growing bacillus is used, so long as the bouillon has been made sufficiently alkaline to prevent the acid produced by the fermentation of the sugar from inhibiting the growth or the bacilli. On the other hand, in Boston, with the same bacillus,

Smith got his best results from the bouillon in which the sugar had been used up by the fermentation of the colon bacillus. Instead of the colon bacilli, yeasts may be added to the soaking meat, which is allowed to stand at about 25° C. The preliminary fermentation of the meat sometimes produces poisonous substances which are deleterious to the horses. When we make toxin for use in the Schick test or in making toxin-antitoxin we do not allow the meat to ferment except through the colon bacilli or some other harmless microörganism. We have obtained our strongest toxin from unfermented fresh veal.

Under the best conditions we can devise, toxin begins to be produced by bacilli from some cultures when freshly sown in bouillon some time during the first twenty-four hours; from other cultures, for reasons not well understood, not for from two to four days. In neutral bouillon containing traces of sugar the culture fluid frequently becomes slightly acid and toxin production may be delayed for from one to three weeks. The greatest accumulation of toxin is on the fourth day, on the average, *after* the rapid production of toxin has commenced. After that time the number of living bacilli rapidly diminishes in the culture, and the conditions for those remaining alive are not suitable for the rapid production of toxin. As the toxin is not stable at 35° C., the deterioration taking place in the toxin already produced is greater than the amount of new toxin still forming.

Bacilli, when repeatedly transplanted from bouillon to bouillon, gradually come to grow on the surface only. This characteristic keeps the bacilli in contact with the oxygen and seems to aid in the development of toxin. With some peptones Wilcox has found that after long cultivation the power to produce toxin formerly held by the culture is greatly lessened while other peptones have no such influence.

Can Virulence be Estimated Alone by Toxin Production?—The virulence of diphtheria bacilli depends on at least two factors: The ability to produce toxin and the ability to infect the mucous membrane epithelium. The virulence of diphtheria bacilli from different sources, as measured by their toxin production, varies considerably. Thus, as an extreme instance, 0.001 c.c. of the filtrate of a forty-hour bouillon culture of our most toxic strain will kill a guinea-pig, which would require 0.1 c.c. of our least virulent strain to kill. This difference does not depend on the unequal growth of the bacilli. The amount of growth should always be observed, as one culture may have fifty times as many bacilli as the other. The other factor is shown strikingly by the fact that when the bacilli of our most toxic strain are washed free of the toxin in a culture they are far less deadly in the guinea-pig than some less toxic cultures. They are not able to withstand the effect of the antibacterial substances of the guinea-pig's tissues. The diphtheria bacilli differ somewhat in the tenacity with which they retain their power to produce toxin when grown outside the body. The bacillus that we have used to produce toxin in our Health Department laboratory has retained its power unaltered for over thirty years. Other bacilli have lessened their capacity for toxin production after being kept six months. Brown and Smith[1] report on a culture in which 5 c.c. was the minimum toxic dose of the filtrate. The passage of diphtheria bacilli through the bodies of susceptible animals does not increase their toxin production to any considerable extent. Although it is difficult to establish the fact, there is probably no doubt that the invasive virulence of

[1] Jour. Med. Res., vol. **30**, No. 3, 443.

the diphtheria bacillus is increased by such passage. This ability to attack the mucous membranes of the respiratory tract is almost certainly kept at its height by growth in actual disease and lessened by remaining quiescent in healthy carriers.

Comparative Toxicity of Bacilli and Severity of Case.—From the severity of an isolated case the toxicity of the bacilli cannot be determined. The presence of slight traces of antitoxin in the blood of the person attacked and the association of other bacteria are at least two of the disturbing factors. The most toxic bacillus we have ever found was obtained from a mild case of diphtheria simulating tonsillitis. Another case, however, infected by this bacillus proved to be very severe.

Virulent Bacilli in Healthy Throats.—Fully toxic bacilli have frequently been found in healthy throats of persons who have been brought in direct contact with diphtheria patients or diphtheria carriers without contracting the disease. It is therefore apparent that infection in diphtheria, as in other infectious diseases, requires not only the presence of toxic bacilli, but also a susceptibility to the disease, which may be local or general. We now know that in large cities 70 per cent. of all persons above the age of six years are protected from infection because of having antitoxin present in their blood. Park and Beebe (1894) made an examination of the throats of 330 healthy persons who had not come in contact, so far as known, with diphtheria, and they found toxic bacilli in 8, only 2 of whom later developed the disease. In 24 of the 330 healthy throats non-toxic bacilli similar to the toxic diphtheria bacillus were found. Very similar observations have since been made by others in many widely separated countries. In 1905 von Sholly in our laboratory examined 1000 throats of those who had not knowingly been in contact with diphtheria and found toxic diphtheria bacilli in 0.5 per cent. of the cases. We have found toxic bacilli in about 5 per cent. of cases of scarlet fever. This indicates they are more prevalent in throats than a single culture from normal cases indicated. The tissue changes due to scarlet fever apparently make the throat more suitable for the growth of the diphtheria bacillus, so that the few bacilli present in many cases increase sufficiently to be caught in the swab and transferred to the culture media. These bacilli do not readily cause diphtheria under ordinary conditions. Last year we watched 30 families in each of which a diphtheria carrier had been detected and no case of diphtheria developed in any of them. Some of the children harboring bacilli, but not developing diphtheria, showed positive Schick tests.

Persistence of Diphtheria Bacilli in the Throat. — The continued presence of toxic diphtheria bacilli in the throats of patients who have recovered from the disease has been demonstrated by all investigators. In the investigations of 1894 we found that in 304 of 605 consecutive cases the bacilli disappeared within three days after the disappearance of the pseudomembrane; in 176 cases they persisted for seven days, in 64 cases for twelve days, in 36 cases for fifteen days, in 12 cases for three weeks, in 4 cases for four weeks, and in 2 cases for nine weeks. Since

then we have met with a case in which they persisted with full toxicity for eight months. It is safe to say that in over 10 per cent. of the cases a few bacilli persist two weeks after the disappearance of the exudate and in over 1 per cent. four weeks. It is extremely difficult to prove that a case is absolutely clear, as the bacilli may remain hidden in the epithelial cells of some tonsillar crypt and not be detected by cultures.

Classification of Carriers.—Those harboring diphtheria bacilli after recovery from disease may be called convalescent carriers; those who carry the bacilli in their throats or adnexa though they have never given evidence of clinical diphtheria may be called contact carriers. These two groups may be transient carriers or chronic carriers. In chronic carriers the removal of hypertrophic lymphatic tissue aids the disappearance of the bacilli.

Diphtheria-like Bacilli Not Producing Diphtheria Toxin.—In the tests of the bacilli obtained from hundreds of cases of suspected diphtheria which have been carried out during the past thirty years in the laboratories of the Health Department of New York City, in over 95 per cent. of cases the bacilli derived from exudates or pseudomembranes and possessing the other characteristics of the Löffler bacillus have been found to be toxic, that is, producers of diphtheria toxin. But there are, however, in inflamed throats as well as in healthy throats, either alone or associated with the toxic bacilli, not infrequently bacilli which, though morphologically and in their behavior on the usual culture media identical with the toxic diphtheria bacillus, are yet producers, at least in artificial culture media and the usual test animals, of no diphtheria toxin. Between bacilli which produce a great deal of toxin and those which produce none we find all grades of toxicity. We believe, therefore, in accordance with Roux and Yersin these non-toxic, but otherwise diphtheria-like, bacilli should be considered as attenuated varieties of the diphtheria bacillus which have lost their power to produce diphtheria toxin. This belief has recently been proved. Toxic diphtheria bacilli follow the habit demonstrated in many species of pathogenic microbes already studied, that of a splitting off of non-toxic varieties. The important practical point is that the great majority of these bacilli have lost permanently their pathogenic properties, and that we treat all non-virulent bacilli as if they were harmless parasites.

A number of observers have held, however, that all the various forms of diphtheria-like bacilli, even including the solidly-staining B. hofmanni (see below), are the result of more or less transitory variations of the same species and hence that the toxic forms are the result of a rapid adaptation to environment and consequent pathogenesis of the non-toxic forms, both typical and atypical. This question is of practical importance in methods of handling the persons harboring these bacilli.

Wesbrook, Wilson and McDaniel[1] long ago made a provisional classification based upon the morphology of the individual bacilli into three groups, called granular, barred and solid—two of the groups into seven types and the other into five, two of the types corresponding with those in the other groups not having been seen. They state that there is generally a sequence of types in the varia-

[1] Tr. Assn. Am. Phys., 1900.

tions which appear throughout the course of the disease, the granular types, as a rule, predominating at the outset of the disease, and these giving place wholly or in part to the barred and solid types shortly before the disappearance of diphtheria-like organisms.

The inference drawn from this work is that the diphtheria bacillus may be rather easily, especially in the throat, converted into non-granular, solidly staining forms of the "pseudodiphtheria" type, and that the converse may occur, and that therefore all diphtheria-like bacilli must be considered a possible source of danger.

In studying the subject Williams[1] (1902) came to the conclusion that non-toxic diphtheria-like organisms retain their characteristics under various artificial and natural conditions, and that they may be regarded from a public health standpoint as harmless. After twenty-five years of practical experience our opinion remains the same.

Among the diphtheria-like bacilli which produce no diphtheria toxin and which are obtained frequently from normal or slightly inflamed throats or from other mucous membranes are some that may be slightly pathogenic for guinea-pigs since they may kill, as we found, in doses of 5 c.c. of broth culture subcutaneously or intraperitoneally injected. Animals are not protected by diphtheria antitoxin from the action of these bacilli, showing that their poisonous action is not due to diphtheria toxin. At autopsy the bacilli are usually found more or less abundantly in the blood and internal organs. These bacilli were first described by Davis[2] from our laboratory.

Immunological Types of Diphtheria Bacilli.—Just as with pneumococci, streptococci and other bacteria, diphtheria bacilli are found to divide themselves into different immunological groups. The table[3] below shows some of these types.

DIRECT AGGLUTINATION OF THE STRAINS OF B. DIPHTHERIÆ USED FOR TOXIN AND ANTITOXIN COMPARISON.

Strains.	Horse serum titer.					
	No. 8.	Nodet.	Sir-beaux.	Benja-min.	11.	3362.
Type I. Our "No. 8"	1600	0	0	0	0	
Type I. Americaine (our "No. 8")	1600	0	0	0	0	
Type III. Nodet	0	1600	0	0	0	0
Type IV. Benjamin	0	0	0	800	800	
Type IV. 11	0	0	0	800	800	
Type V. Sirbeaux	0	0	400	0	0	0
No. 3362	0	0	0	0	0	400

The toxins[2] from these different types of bacilli appear to be identical, that is, the antitoxin developed in animals because of the injection of the toxin of any one of these types will neutralize the toxin produced by any of the others. A monovalent antitoxin suffices for giving protection

[1] Jour. Med. Res., June, 1902, **8**, 83.
[2] Med. News, April 29, 1899.
[3] Park, Williams and Mann: Jour. Immunol., **7**, 243.

as well as for treatment. Occasionally cases considered to be diphtheria have occurred in which antitoxin seemed to have been useless. Two such cases examined by Williams contained massive membranes of hemolytic streptococci in throats as well as diphtheria bacilli. Both cases developed a fatal streptococcus septicemia.

Resistance to Heat, Drying and Chemicals.—The thermal death-point of B. diphtheriæ with ten minutes' exposure is about 60° C., with three minutes 70° C. Boiling kills almost instantly. The bacillus has about the average resistance of non-spore-bearing bacteria to disinfectants. In the dry state and exposed to diffuse light, diphtheria bacilli usually die in from a few hours to a few days, but when in the dark, or protected by a film of mucus or albumin, they may live for months. Thus we found scrapings from dry bits of membrane to contain vigorous bacilli for a period of four months after removal from the throat, and if the membrane had not been at that time completely used, living bacilli could probably have been obtained for a much longer period. On slate- and lead-pencils, toys, tumblers, as well as on paper money, they may live for several weeks, while on metal coins they die in twelve to thirty-six hours. In culture media, when kept at the blood heat, they usually die after a few weeks; but under certain conditions, as when sealed in tubes and protected from heat and light, they retain their life and toxicity for years. The bacillus is not sensitive to cold, for we found about 10 per cent. of the bacilli to retain their vitality and toxicity after exposure for two hours to several hundred degrees below zero. Many of those which died were probably killed by the mechanical effects of freezing. At temperatures just below freezing they may remain alive for a number of weeks.

Transmission of Diphtheria Bacilli.—These have been found on soiled bedding or clothing of a diphtheria patient, or drinking-cups, candy, shoes, hair, slate-pencils, etc. These sources of infection by which the disease may be indirectly transmitted are the less important. The usual source of the bacilli are the discharges of diphtheria patients; the secretions from the nose and throat of convalescent cases of diphtheria in which the toxic bacilli persist, and from the healthy throats of individuals who acquired the bacilli from being in contact with others having virulent germs. When we consider the number of contact healthy carriers, and that it is only the severe types of diphtheria that remain isolated during their actual illness, the wonder is not that so many, but that so few, persons contract the disease. So-called diphtheritic disease in animals and birds is usually, if not always, due to other microörganisms than the diphtheria bacilli. The only exceptions we personally know of are two instances in which cats had diphtheria.

Relation of Bacteriology to Diagnosis.—We believe that all experienced clinicians will agree that there are certain mild exudative inflammations of the throat of similar appearance some of which are accompanied by the diphtheria bacillus while others are not.

The doubtful cases that have the diphtheria bacilli in the exudate, are capable of giving true characteristic diphtheria to others, while

those in whose throats no diphtheria bacilli exist can under no condition give true characteristic diphtheria to others or develop it themselves. It is, indeed, true, as a rule, that cases presenting the appearance of ordinary follicular tonsillitis in adults are not due to the diphtheria bacillus and do not harbor these bacilli. On the other hand, in small children mild diphtheria very frequently occurs with the semblance of rather severe ordinary follicular tonsillitis, due to the pyogenic cocci. Now that we know about 50 per cent. of children in the cities and a lesser per cent. in the country have a fairly constant supply of antitoxin in their blood, we have reason to believe that many of these doubtful cases are simply diphtheria carriers in which the bacilli are taking little or no part in making the lesions. Like any carriers, they may spread the bacilli to others.

The presence of irregular-shaped patches of adherent grayish or yellowish-gray pseudomembrane on some other portions than the tonsils is, as a rule, an indication of the activity of the diphtheria toxin. Restricted to the tonsils alone, the presence of the bacilli is less certain.

Occasionally, in scarlatinal angina or in severe phlegmonous sore throats, patches of exudate may appear on the uvula or borders of the faucial pillars, and still the case may not be due to the diphtheria bacilli.

The very great majority of cases of pseudomembranes of exudative laryngitis whether an exudate is present in the pharynx or not, are due to the diphtheria bacill , but where no exudate is present the laryngitis is generally a part of the lesions of a beginning measles or grippe. In a few of these cases a dual exudate is present in the absence of bacilli. Nearly all membranous affections of the nose are true diphtheria. When the membrane is limited to the nose the symptoms are, as a rule, very slight; but when the nasopharynx is much involved the symptoms are usually grave.

Paralysis following a pseudomembranous inflammation is an almost positive indication that the case was one of diphtheria, although slight paralysis has followed in a very few cases in which careful cultures have revealed no diphtheria bacilli.

The Value of a Bacteriological Examination.—As a rule, cultures do not give us as much information as to the gravity of the case as the clinical appearances, for before the lapse of the twelve hours required for the laboratory report, the extent of the disease usually allows a prognosis. The reported absence of bacilli in a culture must be given weight in proportion to the skill with which the culture was made, the suitableness of the media, the location of the swab reaching the exudate and the knowledge and experience of the one who examined it.

Diphtheria does not occur without the presence of the diphtheria bacilli; but there have been many cases of diphtheria in which, for one or another reason, no bacilli were found in the cultures by the examiner. This is especially apt to occur when the swab does not touch the pseudomembrane or exudate, as in laryngeal diphtheria. In some of these cases later cultures revealed them. The reverse is also true, the presence of diphtheria bacilli in throats without clear clinical

signs of diphtheria in no sense makes it a case of diphtheria. The culture simply reveals the presence or absence of diphtheria-like bacilli. The symptoms indicate whether diphtheria is present. In a convalescent case the absence of bacilli in any one culture indicates that there are certainly not many bacilli left in the throat, but even repeated cultures cannot absolutely prove their total absence, for in some deep tonsillar crypt a few bacilli may remain in the epithelial cells which the swab fails to remove. The physician must have intelligence to use advantageously laboratory findings.

Technic.—*Collection of the Animal Blood Serum and its Preparation for Use in Cultures.* (See Chapter on Media, page 92.)

Swab for Inoculating Culture Tubes.—The swab used to inoculate the serum may be made as follows: A stiff, thin, iron rod, 6 inches in length, is roughened at one end, and about this end a little absorbent cotton is firmly wound. Wood may be used in place of iron. Each swab is then placed in a separate glass tube, and the mouths of the tubes are plugged with cotton. The tubes and rods are then sterilized by dry heat at about 150° C. for one hour, and stored for future use. The iron rods have proved more serviceable for making inoculations than the wooden sticks, especially in young children and in laryngeal cases. The wood, unless very carefully selected, is apt to break and is too thick to use in the nose.

The sterile coagulated blood serum is contained in small metal boxes or in glass tubes. The swabs and media are packed in containers and deposited at stations or sent by mail or express to any place desired.

Directions for Inoculating Culture Tubes with the Exudate.—The patient is placed in a good light, and, if a child, properly held. The swab, while the tongue is depressed with a spoon, is passed into the pharynx (if possible, without touching the tongue or other parts of the mouth), and is rubbed gently but firmly against any visible membrane on the tonsils or in the pharynx, and then, without being laid down, the swab is rubbed on the blood serum, and the portion which has previously been in contact with the exudate is rubbed a number of times back and forth over the whole surface of the serum. This should be done thoroughly, but it is to be gently done, so as not to break the surface of the serum. The swab should then be placed in its tube, and both tubes, thin cotton plugs having been inserted, are reserved for examination or sent to the laboratory or collecting station (as in New York City). If sent to the Health Department laboratories for examination, the blank forms of report which usually accompany each "outfit" should be filled out and forwarded with the tubes.

Where there is no visible membrane (it may be present in the nose or larynx) one swab should be rubbed over the mucous membrane of the pharynx and tonsils, and another in the nasal cavities, and a culture made from these. In very young children it should be remembered that the throat often contains food or vomited matter. This should be cleared away before using the swab to make the bacteriological examination easier. Under no conditions should any attempt be made to collect the material shortly after the application of strong disinfectants (especially solutions of corrosive sublimate) to the throat. Cultures from the nostrils are often more successful if the nostrils are first cleansed with normal salt solution.

Examination of Cultures.—The culture tubes which have been inoculated, as described above, are kept in an incubator at 37° C. for at least twelve hours and are then ready for examination. When great haste is required, even six hours will often suffice for a sufficient growth of bacteria for a skilled examiner to decide as to the presence or absence of the bacilli. The absence of bacilli at this period cannot be relied upon. In primary cultures it is wise to reincubate tubes taken out under fourteen hours in which no bacilli were found. A small

percentage of these will yield positive results after a few hours' further incubation. On inspection it will be seen that the surface of the blood serum is dotted with numerous colonies, which are just visible. No diagnosis can be made from simple in pection; if, however, the serum is found to be liquefied or shows other evidences of contamination the examination will probably be unsatisfactory.

In order to make a microscopic preparation, a clean platinum needle is inserted into the tube and quite a number of colonies are swept with it from the surface of the culture medium, a part being selected where the most suitable growth is found. A sufficient amount of the bacteria adherent to the needle is washed off in a tiny droplet of water previously placed on the glass slide and smeared over its surface. The bacteria on the glass are then allowed to dry in the air. The glass slide is then passed quickly through the flame of a Bunsen burner or alcohol lamp, three times in the usual way, covered with a few drops of Löffler's solution of alkaline methylene blue, and left without heating for five to ten minutes. It is then rinsed off in clear water, dried, and mounted in balsam. When other methods of staining are desired they are carried out in the proper way (see Methods of Staining).

In the great majority of cases one of two pictures will be seen with the oil-immersion lens—either an enormous number of characteristic diphtheria bacilli, with a moderate number of cocci, or a pure culture of cocci, mostly in pairs or short chains. (See Streptococcus.) In a few cases there will be an approximately even mixture of diphtheria bacilli and of cocci, and in others a great excess of cocci. Besides these there will be occasionally met preparations in which, with the cocci, there are mingled bacilli more or less resembling the Löffler bacilli. These bacilli, which are usually of the pseudodiphtheria type of bacilli (see Fig. 121), are especially frequent in cultures from the nose.

In the doubtful case another culture must be made or the bacilli plated out and tested in pure culture.

Instead of the culture media being inoculated, the swab after being rubbed over the infected area may be returned to its glass tube and sent to the laboratory, where cultures may be made from it on Löffler's blood-agar plates from which colonies may be fished the next day.

Direct Microscopic Examination of the Exudate.—An immediate diagnosis without the use of cultures is often possible from a microscopic examination of the exudate. This is made by smearing a slide or cover-glass with a little of the exudate from the swab, drying, heating, staining and examining it microscopically. The results are more uncertain than when the slides are prepared from cultures. The bacilli from the membrane are usually less typical in appearance than those found in cultures, and they are mixed with fibrin, pus and epithelial cells. They may also be very few in number in the parts reached by the swab, or bacilli may be found which closely resemble the Löffler bacilli in appearance, but which differ greatly in growth and in other characteristics, and have absolutely no connection with them. When in a smear containing mostly cocci a few of these doubtful bacilli are present, it is impossible either to exclude or to make the diagnosis of diphtheria with certainty. Although in some cases this immediate examination may be of the greatest value, it is not a method suitable for general use, and should always be controlled by cultures. When carried out in the best manner an experienced bacteriologist may obtain markedly accurate results. Higley, in a series of consecutive throat cases, made the same diagnosis from the direct examination of smears as the Health Department laboratory made from the culture. To get the exudate he used a probe armed with a loop of heavy copper wire which had been so flattened as to act as a blunt curette. He then made thin smears from the exudate. After drying and fixing by heat the smears were stained for five seconds in a solution made by adding 5 drops of Kühne's carbolic methylene blue to 7 c.c. of tap-water. After washing and drying they were stained for one minute in a solution of 10 drops of carbol-fuchsin in 7 c.c. of water. The dilute solution should be freshly prepared. The diphtheria bacilli will appear as dark red or violet rods with blue granules. and their contour. mode of division, and arrangement are manifest.

Animal Inoculation as a Test of Toxicity.—*The So-called Virulence Test.*—It must be remembered that the smear from the culture if positive only demonstrates the presence of bacilli having the morphology of diphtheria bacilli. If positive cultures are obtained from clinically doubtful cases or if they continue for a long time, or if it is a question of a normal carrier, a complete identification should be carried out to release from quarantine those who may be harboring only harmless diphtheria-like bacilli. This determination is spoken of as the "virulence test," or the test for specific toxin production, as the final identification of a suspected diphtheria bacillus rests on the demonstration that it produces a toxin which is neutralized by diphtheria antitoxin.

Technic of Virulence Test.—The first step in the test is the isolation of a pure culture of the bacillus. A number of plates are poured using nutrient agar or ascitic infusion agar (1 part of ascitic fluid to 5 of agar). After hardening, the surface is streaked across with the growth from the Löffler tube, selecting when possible colonies which, in color and size, resemble those of the diphtheria bacillus and also using a general mixture preferably from the drier portions of the serum medium. After incubation for sixteen hours at 37° C., the growth developing along the line of streak should be examined with the microscope using a low-power objective, preferably a No. 2. Diphtheria colonies (see page 378) are most likely to be found at the edges of the streak. These are fished in the usual way by inserting a straight wire between the objective and the agar, the path of the wire and the actual touching of the correct colony being observed with the microscope. Care must be taken that the wire does not touch anything but the colony. The growth is transferred to ascitic fusion broth or Löffler medium depending on the subsequent method of test selected.

If very few bacilli are present in the original culture, inoculations should be made into several tubes of ascitic broth. As the diphtheria bacillus will grow mostly on the surface of such broth, this portion can be used to inoculate plates with success where direct plates fail.

After incubation, the pure cultures are examined by smear to determine whether diphtheria-like bacilli are present. If the examiner has some experience, he can exclude some cultures at once because of their atypical morphology, especially the short solidly staining B. hofmanni. If in doubt, inoculation of glucose-media can be resorted to as a further procedure, since many of these atypical types fail to produce acid in glucose media, whereas B. diphtheriæ does.

After determining that the cultures are pure and morphologically typical, animal inoculation is resorted to. Two methods are available, the subcutaneous and the intracutaneous.

The Subcutaneous Method.—In this method, 1 c.c. of the ascitic broth culture (incubated forty-eight hours) is injected subcutaneously into a guinea-pig, about 250 gms. in weight. A second guinea-pig is injected with same amount of culture to which had been added 50 to 100 units of diphtheria antitoxin. If the first pig dies within two to three days and on postmortem, shows the typical engorgement of the supra-

renals, and local edema already described and the second pig lives, the bacillus is a true toxin producing diphtheria bacillus, otherwise it is not.

The Intracutaneous Method.—The intracutaneous method requires some experience in recognizing the skin lesions. Its advantage is that 4 to 6 cultures can be tested with only 2 pigs.[1] The abdomens of the pigs are denuded of hair by pulling it out. This is done by grasping an area of hair between thumb and finger and exerting a jerk-like pull. Diphtheria antitoxin, about 200 units, is administered to the control pig by intraperitoneal injection. The growth from a Löffler slant is rubbed up in 20 c.c. of salt solution and 0.15 c.c. injected intracutaneously into each pig. The culture used should not be over twenty-four hours old. If the bacillus is a true diphtheria bacillus, it will develop toxin which will produce in the non-immunized pig a definite local inflammatory lesion after twenty-four hours which goes on to superficial necrosis in forty-eight to seventy-two hours. The pig receiving antitoxin will show no lesion.

Although a very few true diphtheria bacilli have been encountered which produce too little toxin to be demonstrable by these methods, they are so infrequent that for practical purposes they can be ignored. If the virulence test is negative, the case may be discharged from quarantine. Lately the practice has been growing to utilize the primary diagnostic cultures for the skin test. If only a moderate percentage of the bacteria are diphtheria bacilli the test will be successful. Other bacteria than the diphtheria bacilli may cause local skin inflammation, but they do not produce necrosis.

Susceptibility to and Immunity against Diphtheria.—An individual susceptibility, both general and local, to diphtheria, as in all infectious diseases, is necessary to contract the disease. Among the predisposing influences which contribute to the contracting of diphtheria when the infection is present may be mentioned living in overcrowded, ill-ventilated and foul-smelling rooms, impure food, certain diseases, more particularly catarrhal inflammations of the mucous membranes, and depressing conditions generally. Under these deleterious influences an infected mucous membrane may become more susceptible to disease. Age has long been recognized to be an important factor in diphtheria. Children within the first six months of life are rarely susceptible, but exceptionally, infants of a few weeks are attacked. The time of greatest susceptibility is during the first and second years. The human infant is now known to receive immunity from its immune mother chiefly by transfer of antitoxin through the placental circulation, but also probably to some degree by the absorption of antitoxin from the colostrum. This transmitted antitoxin is gradually eliminated by the infant so that it is usually entirely lost after six to twelve months. Later in cities most children become carriers of attenuated diphtheria bacilli at some time during the early years and so, absorbing a little toxin form antitoxin, become immune. In the country this occurrence is less likely to happen, since there are fewer carriers and less human contacts.

[1] Rabbits may also be used.

The Persistence in Man's Blood of Homologous and Alien Antitoxin.—Antitoxins and other antibodies disappear more rapidly when introduced into another species than into one of the same species. In man an alien serum must be used in all except exceptional cases.

In our experiments we have found that an injection into guinea-pigs of 10 units of antitoxin produced by guinea-pigs was retained in appreciable amounts for at least six months, while the injection into guinea-pigs of antitoxins made either in the horse or the goat were noticeable to the same extent for only three weeks. In man there is a rather rapid loss of horse-produced antitoxin during the first few days and then a slow loss, becoming more and more gradual until final elimination at the end of ten days to three weeks. The larger the amount of antibodies injected the longer will be the time before the elimination, but no amount is sufficient to prevent the total elimination within four to six weeks.

For a discussion of the nature of antitoxin and the testing of antitoxin, including the flocculation test, see Chapter VIII.

The Detection of Diphtheria Antitoxin in Body.—For over twenty-five years blood has occasionally been withdrawn from children and adults and tested for antitoxin by intracutaneous or subcutaneous injection of a quantity of the serum plus a definite amount of toxin. This was a time consuming test. In 1913 it occurred to Schick[1] that by an adaptation of the Roemer intracutaneous test he could obtain the information in a much simpler way.

The Schick Reaction.—A minute quantity of toxin is injected intracutaneously, and a local reaction follows if there is less than $\frac{1}{30}$ of a unit of antitoxin per cubic centimeter of blood. This amount is sufficient under ordinary conditions to protect against even the mildest attack of diphtheria. The explanation of the test is that when no antitoxin is present in the fluid of the skin the toxin acts on the skin; when antitoxin is present it neutralizes the toxin so that no poisoning occurs. The absence of a reaction therefore indicates the presence of antitoxin.

A standard diphtheria toxin is diluted at first 1 to 10 in 0.5 per cent. phenol; this dilution will keep in the ice-box with little deterioration for at least two weeks. For use further dilutions are made in normal saline, of such strength that 0.1 c.c. contains $\frac{1}{50}$ of the minimum lethal dose for the guinea-pig.[2] This amount is injected intracutaneously on the flexor surface of the arm or forearm. If the injection has been made properly a definite wheal appears which lasts for several minutes. It is of the utmost importance that the injection be made in and not under the skin. We prefer such a dilution that 0.2 c.c. contains $\frac{1}{40}$ M. L. D. This dilution in the larger amount is easier to measure and gives a somewhat more dependable reaction.

A positive reaction appears in twenty-four to thirty-six hours in the

[1] München. med. Wchnschr., 1913, **9**, 2608–2610.

[2] We have found that the buffer solution recommended by Glenny *et al.* (1.5 per cent. in distilled water of following mixture: 57 grams crystal borax, 84 grams boric acid and 99 grams sodium chloride) preserves the strength of the toxin well.

majority of cases, but in some it is delayed as late as seventy-two hours, and is characterized by a circumscribed area of redness and slight infiltration which measures from 1 to 2 cm. in diameter. It persists for seven to fifteen days, and on fading shows superficial scaling and a persistent brownish pigmentation. The test represents a true irritant action of non-neutralized toxin. Pseudoreactions are seen occasionally in young children and more frequently in adults. They are almost never present in very young infants. These are local sensitization phenomena of a general protein character, and can usually be distinguished from the true reaction due to the specific toxin. They appear earlier, usually within twenty-four hours, are more infiltrated, less sharply circumscribed and usually disappear in twenty-four to seventy-two hours. If the reaction is read on the third, fourth or fifth day the pseudoreaction seldom causes difficulty in the interpretation in children, but in older children, and especially in adults, the pseudoreactions may persist as long as the Schick reaction. On fading, they leave a faintly pigmented area which may show superficial scaling.

By the use of this test a large number of individuals have been shown to be naturally immune. By applying the test, therefore, passive immunization with antitoxin of a large number of individuals can be omitted and the disagreeable symptoms of sensitization avoided. Of 400 cases of scarlet fever isolated in an hospital for contagious diseases, showing a negative Schick reaction and receiving no immunizing dose of antitoxin, not one developed clinical diphtheria. Of these cases many were carriers of virulent diphtheria bacilli.

Our results closely parallel those of Schick. In various groups of children and adults we have obtained quite different results. Those who have lived in the most densely populated portions of the city have the largest percentage of immunes. Those who have lived in the country have the smallest percentage as shown in the table.

SUMMARY OF SCHICK TESTS SHOWING SMALLER (CITY) AND LARGER (COUNTRY) PERCENTAGE OF SCHICK REACTIONS.

	Per cent. + Schick.
Birth	15 to 40
1 to 2 years	50 to 80
2 to 4 years	35 to 70
4 to 6 years	30 to 60
6 to 8 years	25 to 55
8 to 10 years	22 to 55
10 to 12 years	21 to 50
12 to 14 years	17 to 50
14 to 16 years	16 to 50
16 to 30 years	15 to 40

From these figures, then, we may assume that the percentage of individuals susceptible to diphtheria is greatest between the ages of one to four years. When a person once develops antitoxin it is apt to be a permanent immunity. Very few giving a negative Schick ever change.

The Subcutaneous Injection of Toxin—Antitoxin as a Substitute for the Schick Test.—Park noted in 1923 that if a 1 c.c. toxin-antitoxin preparation having a toxicity such that 5 c.c., when injected into a guinea-pig, will cause its death sometime between the fifth and tenth day, is injected just beneath the skin of the anterior portion of the arm, there is a reaction similar to the Schick reaction. The result of the injection should be read on the fifth, sixth or seventh day so as to allow the pseudoreactions to subside. The advantage of the subcutaneous injection is that it acts both as a test for immunity, and as an immunizing treatment.

The disadvantage is that in older children and adults there are somewhat more pseudoreactions and that an overneutralized toxin-antitoxin will fail to give reactions.

This test has been substituted for the original Schick test by us in New York City, but we still use the Schick method for the retest.

Active Immunization Against Diphtheria by Means of Toxin-antitoxin Injections.—The use of this immunizing agent is the result of a long series of investigations as to the possibility of producing antitoxin through injection of these mixtures. In 1895 Babes carried out successful experiments in guinea-pigs. For a time beginning with 1897 the horses in the Health Department of New York City, which are used to produce diphtheria antitoxin, were immunized by us at first with toxin-antitoxin mixtures. In 1903 one[1] of us published the records of a number of horses showing that three injections might cause production of several hundred units of antitoxin in each cubic centimeter of serum. Theobald Smith later made a careful study of the subject in guinea-pigs and suggested the toxin-antitoxin injections in children for practical immunization but never tried it.

Behring deserves, therefore, the credit not of the discovery of the method but of actually applying the toxin-antitoxin mixtures for the immunizing of persons against diphtheria. His investigations begun in 1913 were left incomplete because of the war. He did not use the Schick test to determine the success of the injections.

We began to use the toxin-antitoxin in 1914. We utilized susceptible individuals selected by means of the Schick test. Within a year we had injected about 10,000 infants, children and adults in ten different institutions.

The mixture of toxin-antitoxin that we use for immunization contains $\frac{1}{10}$ L+ of toxin + $\frac{75}{100}$ of a unit of antitoxin to each cubic centimeter, and is slightly toxic to the guinea-pig. During the past year diphtheria antitoxin made in the goat or sheep has been used in place of the antitoxin made in the horse. This was done because of the fear of some that children might be sensitized by the minute amount of horse serum used and so be more susceptible to later injections of therapeutic serums. The dose is 1 c.c., and the number of injections three. The injections are made subcutaneously at intervals of seven

[1] Trans. New York Path. Soc., 1903.

days. The local reactions at the site of injection are generally mild; in the older children and adults the redness and swelling are more marked. General symptoms, like malaise, and temperature of 100° to 102° F., are noted in 5 to 10 per cent. of the cases; in a few the temperature reached 104° F. The symptoms last twenty-four to forty-eight hours, and then rapidly subside. Both local and general symptoms were especially evident in some of those who showed a susceptibility to the protein by giving a combined pseudo- and true Schick reaction, but there were no deleterious after-effects.

The retests with the Schick reaction showed that only 30 to 40 per cent. became immune three weeks after the first injection; about 50 per cent. at four weeks, 70 to 80 per cent. at six weeks and 85 to 90 per cent. at twelve weeks. The best results were obtained with the full immunization, consisting of three injections of 1 c.c. each, given at weekly intervals. The duration of the active immunity was studied in a group of children that was followed up for ten years; these cases showed that the active immunity persisted in the great majority for at least that length of time. It is possible that the immunity induced by the injections of toxin-antitoxin starts a continued cellular production of antitoxin which otherwise would have appeared much later in life, or the presence of diphtheria bacilli in the throat may excite the antitoxin production.

From their results Park and Zingher[1] conclude that it is advisable to immunize children soon after the first nine months of life so as to afford them a protection against diphtheria at a time when the disease is most dangerous. In addition such young children, by not having any hypersensitiveness to the bacillus protein, show very mild local and constitutional symptoms after the injections. An immune child population could thus be developed with the result that fresh clinical cases would be prevented and the bacillus-carrier menace would probably soon disappear as a hygienic factor in our communities. Under our supervision Miss Denny watched the effect of the injections in two thousand infants none of whom were over one week old. Absolutely no bad effects were noted. The immunity response is much less than in older infants. Under the supervision of Dr. Schroder and Dr. Zingher the school children in the public schools were Schick tested and those giving a positive reaction injected with three doses of toxin-antitoxin. Early in 1924 it was estimated that at least 550,000 children had been tested and when necessary immunized. No lasting deleterious effect has ever been noted. The diphtheria cases occurring in New York City in 1917 were 12,638; in 1925, 7500. The deaths from diphtheria in 1917 were 1158; in 1926 they were 425. As a part of our investigation we indexed 200,000 school children. Of these 100,000 were tested and when necessary immunized. The other half acted as controls. The reported cases of diphtheria were five times as numerous in the controls as in the treated cases. The severe undoubted cases were all among the controls.

[1] Arch. Pediatrics, 1914.

Toxoid in Place of Toxin-antitoxin.—We have during the past four years been using successfully a very slightly toxic toxoid for active immunization in about 5 per cent. of the children.

In France, Ramon has utilized a toxoid which has no appreciable toxic action. This is called anatoxin. These preparations are made by adding 0.3 per cent. of formalin to the toxin and placing it in the incubators for three to four weeks.

The Time Required for Animals to Produce Antitoxin.—Interesting and parallel results to those in human beings were noted in guinea-pigs and horses. *Guinea-pigs* have no normal antitoxin and are fairly resistant to active immunization with diphtheria toxin-antitoxin, and in that respect they show an almost complete parallelism to the positive Schick cases among human beings. After injections of toxin-antitoxin, an antitoxic immunity develops slowly, reaching its height from the sixth to eighth week. *Horses*, on the other hand, as a rule, correspond to those human beings who are naturally immune in their behavior toward small doses of toxin-antitoxin. They both give a ready response, even after a single injection of toxin-antitoxin, and show a distinct increase in the antitoxin content toward the end of the first week. Occasionally a horse is found that has no antitoxin before treatment; such animals respond slowly to small doses of toxin-antitoxin. It is probable that the tissue cells of the naturally immune human beings and the majority of horses have acquired the property of giving a quick and easy response to the stimulation of diphtheria toxin.

The Use of Diphtheria Antitoxin for Prevention and Cure.—The antitoxic serum used for the treatment of diphtheria is furnished by Departments of Health and biologic plants in a refined and concentrated form, prepared by a precipitation of the antitoxic or "soluble" globulin from the sera of immunized horses. By this process a considerable portion of the serum proteins and the other serum constituents are eliminated, while the antitoxic properties are retained and concentrated. The methods of administration and the therapeutic and prophylactic effects of the refined antitoxin are the same as those of the ordinary serum, the rashes and other deleterious effects sometimes caused by the injection of serum are less apt to follow.

AMOUNT OF ANTITOXIN REQUIRED IN THE TREATMENT OF A CASE.

	Mild cases, units.	Moderate cases, units.	*Severe cases, units.	*Malignant cases, units.
Children up to 50 pounds weight (under fifteen years of age) . . .	3,000–5,000	5,000–10,000	10,000–20,000	15,000–30,000
Children and adults 50 pounds and over in weight	5,000–10,000	10,000–15,000	20,000–40,000	30,000–60,000

* When given intravenously one-half the amounts stated.

Cases of laryngeal diphtheria, moderate cases seen late at the time of the first injection, and cases of diphtheria occurring as a complication of the exanthemata should be classified and treated as "severe" cases.

In all cases a single dose of the proper amount, as indicated in the schedule, is recommended.

It is recommended that the methods of administration be as follows:

Mild cases—intramuscular.
Moderate cases—intramuscular.
Severe cases—intravenous or both intramuscular and intravenous.
Malignant cases—intravenous.

Some point on the surface of the body should be chosen for the injection, where there is an abundance of subcutaneous cellular tissue, the abdomen or infrascapular region. Before the remedy is administered the skin should be sterilized at the point of injection with tincture of iodine or other disinfectant. The syringe should be thoroughly sterilized. It is better not to employ massage over the point of insertion.

The Early Administration of Antitoxin.—*The earlier the remedy is administered, the more certain and rapid is the effect.* In cases of any severity where diphtheria is suspected, and in cases of croup, it is far better to administer the remedy at once, making a culture at the same time, than to delay the treatment until a diagnosis has been made by bacteriological examination. The first injection should be large enough to control the disease. One large dose given early is far more efficacious than the same amount in divided doses.

Severe cases and those in which the administration of antitoxin has been delayed, or cases which are progressive because of an insufficient first dose, should be given a large intravenous injection whenever feasible. In this way the *full value of antitoxin is obtained at once,* whereas the absorption from the subcutaneous injection is so slow that many hours must elapse before any great amount of antitoxin has found its way into the general circulation. For intravenous injection the antitoxin must be warmed to the body temperature and given very gradually. In older children and adults it is given in the vein just above the elbow, but in infants the external jugular vein may be used.

Immunization.—When children or adults have been exposed to diphtheria they may be protected from the disease by the administration of from 500 to 1000 antitoxin units, the smaller dose being sufficient for infants and young children. The protection does not last longer than from two to four weeks. The injection should be repeated if danger of infection persists longer than three weeks. Before immunizing members of a household exposed to diphtheria it is often advisable to determine by the Schick test whether they have natural immunity and are therefore not in danger of contracting the disease.

Danger in Giving Injections of Antitoxin.—About 1 in 10,000 persons develop, within a few minutes after an injection of serum, alarming symptoms. About 1 in 60,000 of the injected die. About 35 deaths in all have been reported. In the first 140,000 persons injected by New York City Health Department Inspectors there have been 2 deaths due

to serum. About the same proportion is reported from Boston. The persons suffering severe symptoms have usually been subject to asthma, while the fatal cases usually have the pathological changes known as status lymphaticus. A few of these rare cases die almost instantly. As a rule, when death occurs it takes place within a few minutes after the development of symptoms. Usually the respiratory rather than the circulatory center seems to be affected. Persons who have not reacted badly to a first injection do not need to fear a second. So far as known all fatal results have followed the first injection.

Mixed Infection in Diphtheria.—Toxic diphtheria bacilli are not the only pathogenic bacteria present in human diphtheria. Various cocci and bacilli more particularly streptococci, staphylococci, pneumococci, and influenza bacilli, are always found actively associated with the bacillus in diphtheria, playing an important part in the disease and leading often to serious complications (sepsis and bronchopneumonia). Investigations indicate that when other pathogenic bacteria are associated with diphtheria bacilli they mutually assist one another in their attacks upon the mucous membrane, the streptococcus being particularly active in this respect, often opening the way for the invasion of the diphtheria bacillus into the deeper tissues or supplying needed conditions for the development of its toxin. In most fatal cases of bronchopneumonia following laryngeal diphtheria we find not only abundant pneumococci or streptococci in the inflamed lung areas, but also in the blood and tissues of the organs. As these septic infections due to the pyogenic cocci are in no way influenced by the diphtheria antitoxin, they frequently are the cause of the fatal termination.

Pseudomembranous Exudative Inflammations Due to Bacteria other than the Diphtheria Bacilli.—The diphtheria bacillus, though the most usual, is not the only microörganism that is capable of producing pseudomembranous inflammations. There are numerous bacteria present almost constantly in the throat secretions, which, under certain conditions, can cause local lesions very similar to those in the less marked cases of true diphtheria. The streptococcus and pneumococcus are the two forms most frequently found in these cases, but there are also others, such as Vincent's bacillus (see below), which, under suitable conditions, excite this form of inflammation, but without serious constitutional symptoms.

Diphtheroids.—There are a large number of small more or less irregular bacilli bearing some morphological resemblance to the diphtheria bacillus which have been loosely termed diphtheroids. They have been found in the nose, throat, eye and other parts of the body, both in health and in disease (Hodgkin's disease, leukemia, etc.). Several of them, *e. g.*, *B. hofmanni*, *B. xerosis*, are considered distinct species (see below). Since this type of organism is so widely distributed, any specific pathogenic properties attributed to them, unless positive proof is offered, must be received with reserve.

Bacillus (Corynebacterium) Hofmanni (Pseudodiphtheria Bacilli).—These bacilli are rather short, plump (Fig. 121) and more uniform in size and

shape than the true Löffler bacillus. On blood serum their colony growth is very similar to that of the diphtheria bacilli. The great majority of them in any young culture show no polar granules when stained by the Neisser method, and stain evenly throughout with the alkaline methylene-blue solution. They do not produce acid by the fermentation of glucose, as do all known virulent and many non-virulent diphtheria bacilli; therefore there is no increase in acidity in the bouillon in which they are grown during the first twenty-four hours from the fermentation of the meat sugar regularly present. They are found in varying abundance in different localities in New York City, in about 1 per cent. of the normal throat and nasal secretions, and seem to have now at least no connection with diphtheria; whether they were originally derived from diphtheria bacillus is doubtful. They have been called *pseudodiphtheria bacilli*, and more properly *B. hofmanni*. In bouillon they grow, as a rule, less luxuriantly than the diphtheria bacilli. Some of the varieties of the pseudodiphtheria bacilli are as long as the shorter forms of the virulent bacilli. When these are found in cultures from cases of suspected diphtheria they may lead to an incorrect diagnosis. These bacilli are found occasionally in all countries where search has been made for them. There are also some varieties of diphtheroids which resemble the short *B. hofmanni* in form and staining, but which produce acid in glucose bouillon.

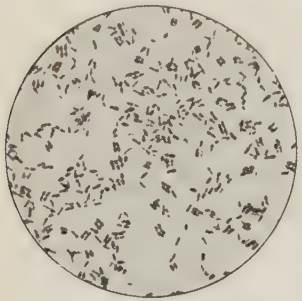


FIG. 121.—Pseudodiphtheria bacilli. (B. hofmanni.)

Bacillus (Corynebacterium) Xerosis.—Diphtheroid bacilli were found by Kutschert and Neisser (1884) in the condition known as xerosis conjunctivæ. Since then they have been found frequently in normal conjunctivæ. These bacilli went by the name of xerosis bacilli. Under this name, no doubt, different observers have placed several varieties of bacilli morphologically somewhat similar to the diphtheria bacilli. Knapp first limited the name to bacilli that ferment saccharose and not dextrin, in contradistinction to diphtheria bacilli which ferment dextrin and not saccharose. The fermentation tests for three of the diphtheria group, according to Knapp, may be shown as follows:

Species.	Dextrose.	Levulose.	Galactose.	Maltose.	Saccharose.	Dextrin.
B. diphtheria	+	+	+	+	−	+
B. diphtheria-like	+	+	+	+	−	+
B. xerosis*	+	+	+	+	+	−
B. hofmanni*	−	−	−	−	−	−

NOTE.—One per cent. sugar in Hiss serum-water media.
* Diphtheroids.

These reactions do not separate the non-virulent diphtheria bacilli from the virulent diphtheria bacilli.

B. Segmentosus (Corynebacterium Segmentosum.)—Cantley,[1] in England, in 1893, described a bacillus which he found in acute rhinitis and called B. coryzæ segmentosus. It has since been found in acute rhinitis in a large percentage of cases by Burnham, Allen[2] and others in England and Walter[3] in this country. It is characterized by a barring due to an unstained septum. It is known in England as B. septus. It has not been minutely enough studied to differentiate it from B. xerosis.

Micro-aërophilic "Diphtheroids."—Certain "diphtheroid" bacilli choose for their most abundant growth that part of the medium which contains only a small amount of oxygen. Thus in a deep shake agar tube culture, a band of growth appears a short distance from the surface of the medium and few colonies appear below this band. Among these microaërophilic bacilli (see page 56) the acne bacillus is of interest to us because of its probably etiological importance in acne vulgaris.

Bacillus Acne (Corynebacterium Acnes.)—A short, rather plump, irregularly shaped, bacillus was first reported as occurring in acne pustules and comedones by Unna[4] in 1894. A morphologically similar bacillus was grown by Sabouraud, in 1897, in pure cultures on an acid glycerin agar. Whether or not this was the acne bacillus remains doubtful, since pure aërobic cultures are so difficult to obtain. In 1907 Halle and Civatte[5] found an anaërobe in the sebaceous follicles from all faces examined. This threw doubt on the etiological relationship of this bacillus to acne. But Gilchrist and others found a similar bacillus most abundantly in acne lesions, though it was frequently accompanied by a small coccus. Fleming,[6] in 1909, found a similar anaërobic diphtheroid in large numbers in acne vulgaris and obtained abundant anaërobic growths on oleic acid glycerin agar.

The bacillus now accepted as *B. acne* has the following characteristics: The cultures are made up of short, rather irregularly club-shaped, non-motile rods averaging 2μ by 0.5μ. These are Gram-positive and take ordinary stains well. They grow best with only a small amount of oxygen; that is, in shake tube cultures the most abundant growth appears in the form of a hazy band about half an inch from the surface of the medium and about a fourth of an inch thick. This develops in about three to five days at 36° C.

In our laboratory a semisolid medium (page 120) has been found to be best for the isolation of this bacillus. A shake culture is made in a tube of this medium. If the tube, after sterilization by carbolic acid, is cut just below the zone of growth and new shake culture tubes made from a portion taken from the periphery of this zone, repeating on subcultures if necessary, a pure culture will finally result.

"Bacillus Typhi-exanthematici."—The "Bacillus typhi-exanthematici" was first isolated and named by Plotz,[7] from the blood of individuals suffering from typhus fever.

[1] Local Government Reports, 1894, 5.
[2] Lancet, November, 1908, p. 1591.
[3] Jour. Am. Med. Assn., 1910, **55**, 1091.
[4] Histopathology of Diseases of the Skin, 1894.
[5] Ann. de Dermat. et de. Syph., 1907, 184.
[6] Lancet, 1909, i, 1207.
[7] Jour. Am. Med. Assn., 1914, **62**, 1556; La Presse méd., 1914, **43**, 411.

The organism is a small, slender bacillus, the average length being about 1 micron. The ends are slightly pointed, less often rounded. (Fig. 122.) The organism in young subcultures is Gram-positive. When first isolated it is an obligate anaërobe.

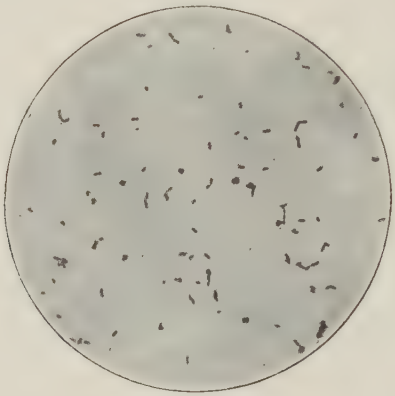


Fig. 122.—"B. typhi-exanthematici." (Plotz.)

The evidence of any relationship of this microbe to typhus has lost much of its significance since the finding of abundant agglutinins against one of the proteus group of organisms (see Filtrable Viruses), and the finding of antibodies against the Plotz bacillus in cases other than typhus. The further studies on the Rickettsia also give other evidence.

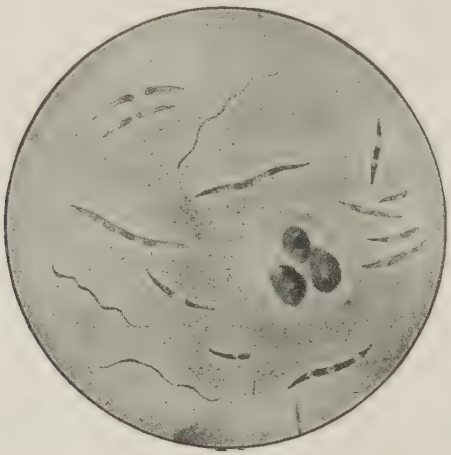


Fig. 123.—Vincent's bacillus with accompanying spirochetes.

Bacillus Fusiformis (**Fusiformis Dentium**).—This organism together with spirochetes was found by Plaut and Vincent in pseudomembranous inflammation of the throat (Vincent's Angina). The constancy of its presence and the preponderance of its numbers in smears from this condition suggest strongly its etiological connection with the disease. Fusiform bacilli and spirochetes are also encountered in gangrene, noma, ulcerative stomatitis, gingivitis, dental caries and even around the gum margins of dirty teeth, especially if there are

deposits of tartar. Although B. fusiformis is present in many conditions, either because of the greater virulence of certain strains or because of reduced resistance of the tissues it, either with or without spirochetes, is probably the essential agent in the production of Vincent's angina and noma and acts as a contributing factor in other lesions.

The fusiform bacilli are anaërobic and have been cultivated. Very successful methods of isolation and cultivation are those of Krumwiede and Pratt,[1] who studied fifteen strains from various conditions. The typical bacillus is double-pointed, containing one or more granules, which are well demonstrated by Giemsa's stain. In culture the morphology is variable. In fluid media there is a tendency to produce filamentous types, which may form tangled thread-like masses. The colonies are characterized by thread-like outgrowths. The fifteen cultures studied fell into saccharose fermenting and saccharose non-fermenting groups, but this difference had no correlation to the source of the culture. The fusiform bacillus and the spirochetes accompanying it in the lesion were never encountered together in cultures made from single colonies, although Tunnicliff claimed that the fusiform bacillus and the spirochete were only different forms of the same organism. Pratt found no serological-likenesses in a series of cultures.[2]

The microscopic recognition of B. fusiformis may be made in direct smears from the lesion. The differential diagnosis between the short, more deeply staining club-like forms and B. diphtheriæ, as it often appears in direct smears stained by alkaline methylene-blue solution, may be made by staining by Gram's stain, which shows B. fusiformis as mostly negative to Gram, with faintly staining granules.

[1] Jour. Inf. Dis., 1913, **12**, 199; 1913, **13**, 438. See also Varney: Jour. Bacteriol., 1927. **13**, 275.

[2] Jour. Inf. Dis., 1927, **41**, 461.

CHAPTER XX.

MICROÖRGANISMS OF THE ALIMENTARY CANAL AND THEIR CONTROL.

Conditions Influencing Microbal Development.—The alimentary tract both as regards foodstuff, varying degrees of oxygen tension and reaction gives so many different conditions for growth that numerous varieties of microörganisms find an optimum environment at some level. Thus, the mouth offers, in general, an aërobic area, although with its various crevices between the teeth, in the folds of the mucous membranes and the crypts of the tonsils, anaërobic types may flourish with aërobic types, which absorb the oxygen. In the stomach, when the acidity is normal, most germs are killed unless protected by food particles. As soon as the duodenum is reached the oxygen tension becomes low, and from there on an anaërobic condition exists. More aërobic conditions are reached in the lower colon and rectum. It is evident that the degree of digestion of the food and the proportion of unabsorbed carbohydrate and protein at different levels will also influence the flora, likewise the products of bacterial growth at one level will influence the growth of other types at this and at lower levels. As the intestinal contents pass downward they carry with them the flora from a higher level, but unless the conditions are particularly favorable for their growth they are probably quickly overshadowed by the more adapted types. In the lower part of the large intestine and in the rectum, due to the gradual loss of water, there is marked tendency of many of the microörganisms in feces to die or to become so attenuated as to be incapable of further growth. It is estimated that at least 5 to 8 grams of bacteria, mostly dead, are excreted each day with the feces.

Development of the Intestinal Flora.—At birth the meconium is sterile unless fetal infection has taken place due to general infection in the mother. Shortly after birth chance bacteria such as staphylococcus, B. subtilis and yeasts are found entering either through the rectum or by way of the mouth from swallowing saliva or food. Very soon, through further ingestion of microbes and by multiplication of different types at their optimum levels, a more or less distinctive alimentary flora is established, distinctive in that the breast-fed infant, the artificially-fed infant and the adolescent or adult each has, considering the dominant types, a somewhat characteristic flora. This flora is susceptible, however, to variation through the administration of cathartics, by prolonged changes in diet, through feeding of cultures or during alimentary infections. The possibility of implantation of microörganisms by feeding is considered below.

Importance of the Intestinal Flora.—Whether the development of such an intestinal flora is of physiological advantage to the host is open to question. Successful experiments in raising animals with a sterile intestinal tract show that such a flora is not a physiological necessity. Experiments in which animals so raised did not do well cannot be considered evidence against this view, as the conditions of the experiment may have deleteriously influenced development. Undoubtedly the character and balance of the flora does act as a protection under some circumstances, being antagonistic to the implantation of an exogenous pathogenic type. Abnormal variations, however, in balance between the dominant types may be the basis of intestinal symptoms (see below). In one sense, part of the intestinal flora is a potential menace to the host. The intestinal mucosa is a partial obstacle to the passage of the microbes into the tissues, lymph and blood. A few, however, evidently escape as the fairly frequent localization in the gall-bladder, kidney and elsewhere show. Probably this occurs constantly in small numbers, but because of the slight virulence of the majority of such types or due to the resistance of the host or both they are promptly disposed of.

FIG. 124.—B. bifidus, representing the various forms described; the irregularly stained or vesicular forms being from old cultures. × about 1800 diameters.

Dominant Intestinal Bacterial Types.—B. (Bacterioides) bifidus.—This anaërobe was isolated by Tissier from the stools of breast-fed infants and from the superficial ducts of the mammary glands of mothers. It is Gram-positive or contains a Gram-positive granule with a Gram-negative body. In stools it is a slender bacillus with one end tapering, the other club-shaped. In cultures it has the property of the developing bifid ends. It produces acid freely but no gas from lactose or other sugars.

B. (Lactobacillus) Acidophilus.—(See p. 407.)

Enterococcus or Micrococcus Ovalis.—This is the term commonly applied to the streptococci found in the healthy intestinal tract. They

are Gram-positive oval or rounded cocci, usually in pairs or in short chains. Capsules may be demonstrable. Due to delayed cleavage, pseudobacillary forms may develop on certain media. The cocci are aërobic, facultative anaërobic, non-liquefying, markedly heat-resistant, usually ferment lactose and mannite and frequently coagulate milk. These cocci are probably related to the common milk-souring streptococci and to the streptococci of the upper respiratory tract. On blood-agar plates they show either the alpha or gamma characteristics.

Spore Bearing Anaërobes.—The characteristics of B. welchii, B. sporogenes and B. putrificus are described in Chapter XXXIII.

Spore Bearing Aërobes.—B. mesentericus, B. cereus and many similar varieties are found.

B. Coli and Allied Types.—See Chapter XXI.

The intestinal flora has been studied chiefly through the feces and on experimental animals. Occasionally intestinal contents have been obtained from man during laparotomies or from fistulæ. The Einhorn tube opened a new mode of obtaining material.[1]

Intestinal Flora of Breast-fed Infants.—The streptococcus (micrococcus) types preponderate in the duodenum and are most numerous when digestion is in progress. The dominant type in the remainder of the small intestine is B. aërogenes, B. coli being most numerous about the cecal level. B. bifidus finds its optimum in the large intestine. Putrefactive bacteria are uncommon in normal breast-fed infants. Fecal smears show a preponderance of Gram-positive types.

Intestinal Flora of Artificially-fed Infants.—The Gram-negative B. coli and B. aërogenes as well as the Gram-positive streptococci are relatively increased as compared with the breast-fed infant, while B. bifidus types are diminished, being replaced by colon types and B. acidophilus. Proteolytic action is evidenced in the alkaline reaction of the feces. In general the distribution of the types follows that in breast-fed infants. The character of the flora is susceptible to variation according to the balance of carbohydrate to protein and by changes in the carbohydrate employed.

Intestinal Flora of Adults.—The duodenum except during digestion has a low bacterial content. Cocci usually predominate in the upper small intestine. B. coli types become numerous in the lower small intestine, and predominate about the cecal level. Spore bearing aërobes, usually strongly proteolytic are common in the lower intestine. The varieties present correspond to some of the varieties that are present in water and milk of the vicinity. These and B. welchii and to a limited extent the proteolytic anaërobes, B. acidophilus and certain yeasts, as well as B. proteus, B. subtilis, molds and spirilli, constitute the flora of the large intestine. The flora as compared with that of infants is characterized by the relative suppression of essential carbohydrate-fermenting types. The carbohydrate of adult life is mostly starch and the products

[1] For studies with intestinal tube or similar methods see: Hess, Ergeb. d. Inn. Med. u. Kinderh., 1914, **13**, 530. Aronowitch, Coleman and Einhorn: Proc. Soc. Exp. Biol., 1922, **20**, 97. V. der Reis: Klin. Wchnschr., 1922, **1**, 257. Goldman: Jour. Inf. Dis., 1924.

of its cleavage are probably quickly absorbed, leaving the more slowly cleaved and absorbed protein as available foodstuff for the bacteria. The colon types and the aërobic and anaërobic proteolytic types which can utilize this material therefore dominate the flora. Although the relative preponderance as described holds true, it should be emphasized that each bacterial variety can be isolated from any level.

Variations in the Bacterial Flora and Their Significance.—The flora as given for the various types is basically dependent on the diet. Thus, in the nursing infant, the carbohydrate is sufficient in amount to supply the conditions for growth of the essentially fermentative types. These because of their acid production limit to a marked degree the multiplication of other types, especially the proteolytic types. Although in the case of the artificially fed infant the available carbohydrate is less in amount, it is still sufficient to lead to relatively the same result. If, however, the protein proportion of the food be increased, a relative or complete suppression of acid-producing types with an increase of the proteolytic types occurs, approximating the flora of the adult. The activity of B. coli as regards end-products depends also on the availability of carbohydrates. If present in sufficient amounts, lactic acid and other end-products of carbohydrate fermentation are produced. When absent, the end-products of protein cleavage, H_2S,NH_3, etc., are produced. It is easy to see how carbohydrate fermentation acts as a check to excessive putrefactive activity, which condition may be deleterious to the host. The bacterial flora, as a rule, is little influenced by the ingestion of bacteria in the foods. Starvation, however, will markedly reduce the number of bacteria in the upper level[1] of the small intestine.

When infection occurs, such as dysentery or cholera, these organisms and others similar to them may dominate the intestinal flora and a specific disease develops. Apart from such well-known types of disease, there are other morbid conditions which are intimately associated with changes of the intestinal flora, even if these changes are not of etiological importance. They may be characterized by a marked increase of (*a*) putrefactive bacteria accompanied by the end-products of proteolysis and the presence of indican in the urine; (*b*) bacteria causing carbohydrate fermentation and a consequent diarrhea; (*c*) pyogenic cocci, probably due to inflammatory areas in the intestinal mucosa, which areas may serve as foci for dissemination and infection of joints, etc. The cocci may have been injested, arising from chronic infection of tonsils respiratory tract, etc.[2]

On this basis attempts have been made to influence these conditions. Cathartics, intestinal antiseptics and starvation have no appreciable beneficial value. Changes in diet so adapted as to limit or supply the

[1] For discussion of effect of fasting and of local antiseptics see: Cushing and Livingood: Johns Hopkins Hosp. Repts., 1900, **9**, 543. Sisson: Am. Jour. Dis. Child., 1917, **13**, 117. V. der Reis: Klin. Wchnschr., 1922, **1**, 950. Dragstedt, Dragstedt and Nisbet: Jour. Lab. and Clin. Med., 1922, **8**, 190.

[2] For discussion of possible relationship of intestinal streptococci to arthritic conditions, see: Mutch: New York Med. Jour., 1921, **113**, 713; Lancet, 1921, p. 1266.

optimum foodstuff for certain types is apparently the most important controlling factor. An increase of carbohydrates may lead to a flora dominated by B. acidophilus types. Not all carbohydrates, however, have an equal tendency to bring about this transformation to a dominant fermentative flora. Lactose and dextrin are most effective (Torrey). Proteins likewise differ in their effect on the putrefactive flora. Those of milk are less likely to increase this flora than those of meat. Vegetable proteins apparently do not encourage an increase of these types. Metchnikoff's sour-milk therapy was an attempt to modify the flora through the introduction of exogenous lactic acid bacteria and their products.

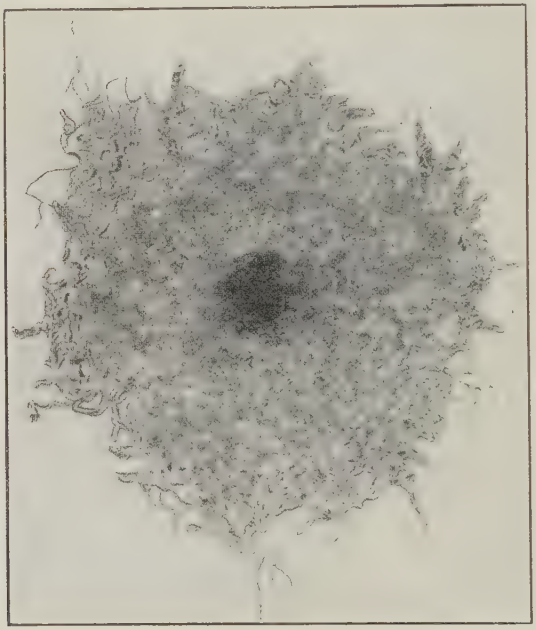


FIG. 125.—B. bulgaricus; seventh day (44°) colony. Whey agar plate. × 50 diameters. (White and Avery.)

Types of Sour Milk (Lactic Acid Milks).—Milk will sour naturally due to lactic acid bacteria which are contaminations of the milk from its surroundings. Buttermilk is a naturally soured product. Metchnikoff advised the use of B. bulgaricus (see below) found in the sour milk of Eastern Europe and in Asia. Many lactic acid-milk preparations are available. To control the appearance of the end-product and to have a uniformly palatable preparation, it is necessary to heat the milk and add a starter. Thus "koumyss" is fermented by lactic acid bacteria and yeasts, "Maadsoun" and "zoolak" by B. bulgaricus and lactic-acid-producing streptococci and diplococci. Many milk dealers now offer lactic acid milks under various trade names, the starters used being those mentioned in various combinations. B. bulgaricus alone

gives too sour a milk. There is no evidence that B. bulgaricus may be implanted in the intestinal tract, which Metchnikoff thought probable. The beneficial effects resulting from the use of the above preparations is apparently due to the milk as such, to the acids, with palatability a probable added factor.

The Lactobacilli.—To this group belong B. (Lactobacillus) bulgaricus (Grégeroff), Bacillus (Lactobacillus) acidophilus (Moro) (Lactobacillus acidophil-aërogenes, Torrey and Rahe) and many other varieties. They are gram-positive, non-motile rods, without endospores, growing singly, in pairs or in chains. Many but not all coagulate milk. They are, as a rule, microaërophilic, aciduric and glycophylic. Bacillus acidophilus is the type normally present in small numbers in the normal intestine of man. Two types of colonies on agar have been described (Hull and Rettger), an X type resembling that of Bacillus bulgaricus and smooth edge Y type. These are colony variants. Lactobacillus acidophilus, unlike Bacillus bulgaricus, ferments maltose.

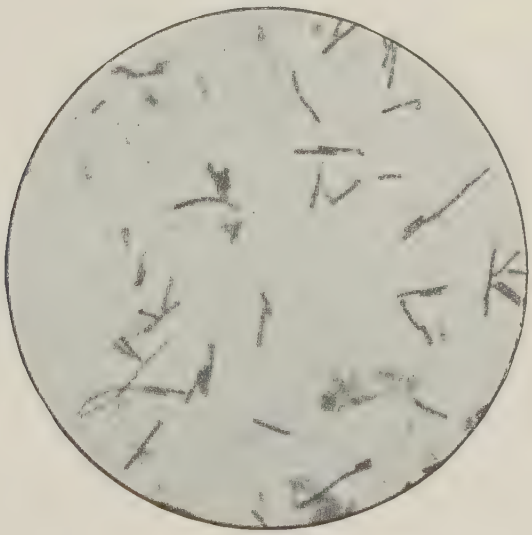


FIG. 126.—B. bulgaricus. × 1000 diameters. (Piffard.)

Since Bacillus bulgaricus appears not to survive after ingestion while Lactobacillus acidophilus is normally present in the intestinal tract, Lactobacillus acidophilus is now used generally in preparations (milk, capsules, broth) intended to be fed for therapeutic purposes. As noted more fully below, there is still some doubt as to what degree implantation occurs. A recently isolated culture is preferred. A wide variety of morbid conditions, obstinate constipation, putrefactive diarrhea, as well as systemic conditions ascribed to toxic products of alimentary tract origin, have been treated by the combined feeding of Lactobacillus acidophilus and carbohydrate. Usually lactose is used as it is not com-

pletely absorbed from the upper small intestine. In suspected pyogenic infection of the colon rectal injections have been used. The reports indicate a high percentage of beneficial effect. The good effects have been ascribed: (*a*) To the acidity produced in the large intestine by the fermentation of the carbohydrates by Lactobacillus acidophilus, the acidity suppressing the proteolytic bacteria and turning the colon varieties to purely fermentative activity (Kendall and Torrey); (*b*) to the implantation of Lactobacillus acidophilus and the crowding out of rival bacteria (Rettger and Cheplin). The general belief is that a true implantation of the ingested types takes place. Work done by Otto in this laboratory raises the question as to whether an alien strain is often implanted. The probability of the acidophilus milk aiding in the multiplication of a resident variety cannot be excluded. How far this is a factor in the final result must rest on further work.

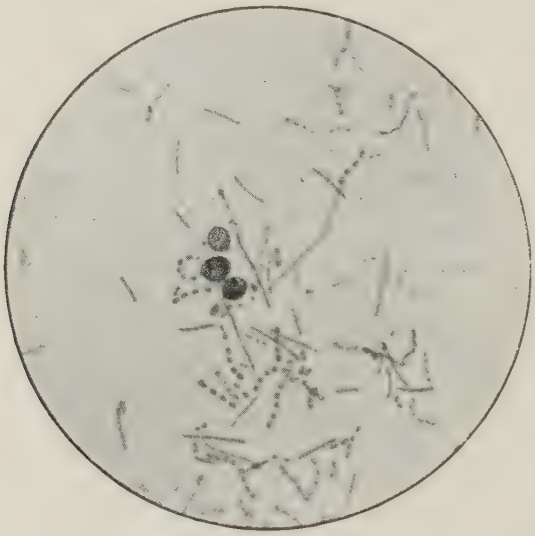


FIG. 127.—"Lactic acid" milk containing B. bulgaricus and a lactose-fermenting streptococcus.

Commercial preparations of Lactobacillus acidophilus in milk are at present the most efficacious. It is probable that agar preparations in the near future will be so improved as to be equally efficacious so far as the number of viable bacteria is concerned. Both preparations should be used within the time stated. Not all commercial milk preparations are alike as regards numbers and variety of bacilli.[1]

The Influence of Available Foodstuffs in Specific Intestinal Infections.—In infections due to members of the typhoid-dysentery group or to the cholera vibrio the presence or absence of available carbohydrates may have an important bearing on the products developed by these bacteria

[1] Kopeloff: Jour. Am. Med. Assn., 1928, **91**, 1187.

and thus on the disease. Each will probably utilize carbohydrate if available, in preference to proteins (protein sparing). There is no evidence that the acids produced from carbohydrate cleavage are any more harmful than those produced by any non-pathogenic fermentative organism. Furthermore, the acid produced would limit their further growth. Not only may the products of protein cleavage be harmful in themselves, but the toxicity of the products of growth, whether a free toxin is produced or not, is enhanced in amount when growth is due to the utilization of protein material.

A free carbohydrate diet, therefore, should be valuable in these diseases not only to spare protein but as an incentive to increased activity of the aciduric types which would aid by limiting multiplication. Although difficulties due to inflammatory conditions of the intestines and other causes may interfere with the efficiency of this mode of treatment, its results in general have been good. Coleman and Schaffer, with the high calory diet in typhoid fever, have demonstrated not only that the nitrogen and weight loss is prevented to a large extent, but also that the toxemia is reduced.[1]

Dental Caries.—There is an increasing evidence that members of the acidophilus group are of importance in the decalcification process in dental caries. The bacilli encountered are apparently different from the acidophilus varieties found in the intestine. The term Lactobacillus odontolytic has been applied to the varieties found in caries. In general it would seem that these high acid producers are the commonest agents in breaking down the dense enamel and the dentin. Probably less active types could decalcify the latter.[2]

[1] For fuller discussion and bibliography see:
Goldman, Jour. of Inf. Dis., 1924.
Herter: Bacterial Infections of the Intestinal Tract, New York, 1907.
Kendall: Am. Jour. Med. Sci., 1918, **156**, 157.
Kopeloff: Lactobacillus Acidophilus, Williams & Wilkins, 1926.
Rettger and Cheplin: A treatise on the transformation of the intestinal flora with special reference to the implantation of B. acidophilus, Yale Univ. Press, 1921.
Torrey: Jour. Infect. Dis., 1915, **16**, 72.
Torrey: Jour. Med. Res., 1919, **39**, 415.

[2] See Appleton: Bacterial Infection, with Special Reference to Dental Practice, Lea & Febiger, 1925. Bunting, Nickerson and Hard: Dental Cosmos, 1926, **58**, 931.

CHAPTER XXI.

THE COLON-TYPHOID GROUP OF BACILLI.

THERE are a number of varieties of bacilli normally occupying the intestines of man and animals which, because they have similar characteristics and live in the colon, are generally grouped together as colon bacilli. Many of the varieties occurring in animals are culturally like those found in man. These bacilli are only pathogenic under unusual conditions. The specific pathogens of the typhoid-paratyphoid group, including the types responsible for food infection, and the dysentery group, also have resemblances among themselves and with the colon bacilli and are often classed together as the typhoid-colon group.

The chief characteristics common to this whole group are: (1) a similar morphology, *i. e.*, short, rather plump non-spore-bearing rods with a tendency to thread formation; (2) a Gram-negative staining reaction; (3) similar growths on agar and gelatin; (4) non-liquefaction of gelatin (a few closely related organisms, such as *B. cloacæ*, liquefy gelatin very slowly).

In order to see more clearly the main points of difference among the subdivisions of this great group the following tabulation of the more important types may be studied. (For names recommended by the S. A. B. and for other differential points, see large table opposite p. 151.)

GROUP OF COLON-TYPHOID BACILLI.

Coli-ærogenes group	B. (coli) communis B. (coli) communior B. (acidi) lactici B. aërogenes types	Normal inhabitants of the intestines, under certain conditions become pathogenic.
Paratyphoid enteritidis group	B. paratyphosus, A B. paratyphosus, B B. (pestis) caviæ B. suipestifer (Hog cholera group) B. enteritidis	Pathogenic for man.[1]
	(Similar types, intermediates between colon and typhoid have been isolated from feces, water and other sources; some are pathogenic.)	
B typhosus		Pathogenic for man.
Dysentery group	B. dysenteriæ (mannite not fermented). B. paradysenteriæ types (mannite fermented).	Pathogenic for man, paradysenteriæ usually less so than dysentery type.
Alkaligenes group	B. alkaligenes.	Occasionally pathogenic.

[1] See page 439 for animal pathogens.

ESSENTIAL DIFFERENTIAL REACTIONS—COLON–TYPHOID GROUP.

- *Lactose*
  - *Acid and gas* (colonies, red on Endo and Conradi)
    - *Glucose—acid and gas—Colon group.* (Russell medium—slant, acid butt, acid and gas.)
  - *not fermented,* (colonies, colorless on Endo, blue on Conradi)
    - *Glucose—acid and gas—Paratyphoid-enteritidis group.* (Russell medium—slant, unchanged butt, acid and gas.)
    - *Glucose—acid only—B. typhosus—dysentery group.* (Russell medium—slant, unchanged butt, acid only.)
    - *Glucose—not fermented—B. alkaligenes.* (Russell medium—slant, unchanged butt, unchanged.)

Among the non-lactose fermenters motility or its absence is of differential value. The fermentative characteristics of value in differentiating members of the subgroups are given with each subgroup.

Russell's Medium.—The explanation given by Russell[1] of the reactions which develop was that the bacilli growing aërobically, that is, on the slant, only utilize the carbohydrate when present in amounts over 0.1 per cent., whereas growing anaërobically stabbed in the butt, they must utilize the carbohydrates for their oxygen supply and therefore ferment the trace of glucose present. Wheeler[2] believes that the glucose in the slant is fermented but that because of the access of oxygen sufficient alkali is formed to keep the indicator from changing color. The butt remains acid because in the absence of oxygen little alkali is formed Krumwiede[3] advises a triple sugar medium, made by adding 1 per cent. of saccharose which serves to exclude many of the intermediate varieties which resemble the paratyphoid types.

THE COLON GROUP.

The first description of an organism of the colon type was by Emmerich (1885), who obtained it from the intestinal discharges of cholera patients. A similar organism was found by Escherich (1886) in the feces of healthy infants. He gave it the name of *Bacterium coli commune.* It has since been demonstrated that closely allied types of bacilli are normal inhabitants of the intestines of most of the lower animals. They are transferred through the feces as manure and sewage to cultivated land, surface waters, etc. During warm weather they may multiply outside of the animal body. Those strains having the chief cultural characteristics of the original strains are classed as colon bacilli, while those differing considerably from it are, while considered in the general group, giving different names, such as paracolon, "atypical coli,"

[1] Jour Med. Res., 1911, **25**, 227.
[2] Jour. Inf. Dis., 1924, **34**, 13.
[3] Jour. Med. Res., 1917, **37**, 225.

etc. These types are especially common in water and their atypical reactions are often due to the suppression of certain characteristics due to unfavorable environment.

The colon group is divided into four main subgroups, *B. communis*, *B. communior*, *B. aërogenes*, and *B. (acidi) lactici*. These subgroups have numerous varieties.

The colon group has interest not only because it excites disease at times in man and animals, but also because it is an index of fecal pollution from man or animals. If from man it indicates the possibility of infection with the typhoid or dysentery bacilli. For the significance of the colon group in water see Bacteriology of Water.

B. (Escherichia) Communis.—This organism is taken as the *type* of the group in the following description. Only differentiating points are noted in the other varieties.

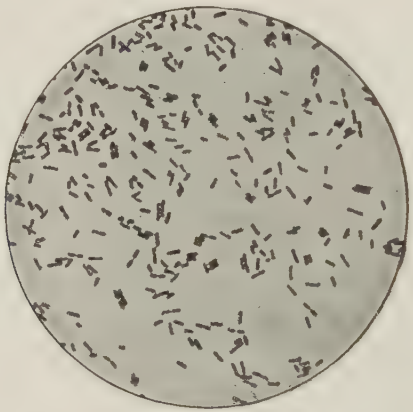


Fig 128.—Colon bacilli. Twenty-four-hour agar culture. × 1100 diameters

Morphology.—*B. communis* varies in morphology. The typical form (Fig. 128) is that of short rods with rounded ends, from 0.4μ to 0.7μ in diameter by 1μ to 3μ in length; sometimes, especially when the culture media are not suitable for their growth and in tissues, the rods are so short as to be almost spherical, resembling micrococci in appearance, and, again, they are somewhat oval in form or are seen as threads of 6μ or more in length. The various forms may often be associated in the same culture. The bacilli occur as single cells or as pairs joined end-to-end, rarely as short chains. There is nothing in the morphology of this bacillus sufficiently characteristic for its identification.

Flagella.—Upon some varieties seven or eight peritrichic flagella have been demonstrated, the non-motile types show none. The flagella are shorter and more delicate than those characteristic of the typhoid bacilli.

Staining.—The *B. communis* stains readily with the ordinary anilin colors; it is always decolorized by Gram's method. Under certain conditions the stained bacilli exhibit bipolar granules.

Biology.—It is an *aërobic, facultative anaërobic, non-liquefying* non-spore-bearing bacillus. It develops best at 37° C., but grows well at 20° C., and slowly at 10° C. It is usually motile, but the movements in some of the cultures are so sluggish that a positive opinion is often difficult. In fresh cultures, frequently only one or two individuals show motility.

Cultivation.—The *B. communis* develops well on all the usual culture media. Its growth on them is usually more abundant than that of the typhoid bacillus or the dysentery bacillus, but the difference is not sufficient for a differentiation.

Gelatin.—In gelatin plates, colonies are developed in eighteen to thirty-six hours. They resemble greatly the colonies of the typhoid bacillus, except that many of them are somewhat larger and more opaque. (See Figs. 50 to 52, page 140). When located in the depths of the gelatin and examined by a low-power lens they are at first seen to be finely granular, almost homogeneous, and of a pale yellowish to brownish color; later they becomelarger, denser, darker, and more coarsely granular. In shape they may be round, oval, or whetstone-like. When the gelatin is not firm the margins of many colonies are broken by outgrowths, which are rather characteristic of colon bacilli.

In stab cultures in gelatin the growth at first takes the form of a nail with a flattened head, the surface extension generally reaching out rapidly to the sides of the tube.

Nutrient Agar.—In plate cultures: Surface colonies are mostly circular, finely granular and rather opaque. The deep colonies are likely to have protuberances. In streak cultures an abundant soft, white layer is quickly developed, but the growth is not characteristic.

Bouillon.—In bouillon the *B. communis* produces diffuse clouding with sedimentation; in some cultures a tendency to pellicle formation on the surface is occasionally seen.

Potato.—On potato the growth is rapid and abundant, appearing after twenty-four to thirty-six hours in the incubator as a yellowish-brown to dark cream-colored deposit covering the greater part of the surface. There are considerable variations from the typical growth; there may be no visible growth at all, or it may be scanty and of a white color. These variations are often due to variations in the potato.

Milk.—Milk is usually coagulated in from one to four days at 37° C. Coagulation is due to the production of lactic and acetic acids. Some stains produce acid, but coagulation does not occur. Usually, however, sufficient acid is produced so that coagulation occurs if the culture is heated to boiling. It is possible that a lab ferment is partly responsible for the coagulation.

Chemical Activities.—Behavior Toward Carbohydrates.—In cultures of *B. communis* many carbohydrates are fermented with production of acid and gas.

Gas Production.—When *B. communis* is grown in a solution of glucose (dextrose), CO_2 and H_2 are produced, in the proportion of $1CO_2$ to $1H_2$ up to $1CO_2$ to $2H_2$. Anaërobic conditions aid gas formation. Very slight

traces of gases other than H_2 and CO_2 are produced. The amount of gas varies in different varieties; the closed arm of the tube half-filled and the H_2 and CO_2 in the proportion 2 to 1 is the characteristic type.

The fermentative reactions of the main subgroups based on the work of Smith[1] and of MacConkey[2] are as follows:

B. (coli) communis—saccharose, negative—dulcit, positive.
B. (coli) communior—saccharose, positive—dulcit, positive.
B. (acidi) lactici—saccharose, negative—dulcit, negative.
B. aërogenes—saccharose, negative—dulcit, positive.

Effect of B. Communis on Nitrogenous Compounds.—*Indol Formation.*—*B. communis* does not liquefy gelatin nor peptonize any albumins. It does, however, break down some of the higher nitrogenous compounds into smaller atom groups. The first noted of these compounds was indol. This is one of the most important products of colon activity, although some varieties lack the ability to produce it. The maximum amount of indol is present about the tenth day.

Reduction Processes.—Nitrates are reduced to nitrites and from the latter ammonia and free nitrogen are derived. Litmus and other dyes are also reduced.

Toxins.—The bodies of dead bacilli contain pyogenic and other substances, which, injected into the circulation, produce paralysis of the striped muscle fibers, convulsions, coma and death. Extracts from some cultures produce irritation of the mucous membranes of the large intestines with dysenteric symptoms.

Effect of Growth on Other Bacteria.—The *B. communis* as well as other members of the colon group act antagonistically to many of the proteolytic bacteria in the intestinal tract, and so inhibit alkaline putrefaction otherwise caused by the latter.

Reaction to High and Low Temperatures.—*B. communis* is killed at 60° C. in from five to fifteen minutes. Frozen in ice a large proportion die, but some resist for six months. Frozen in liquid air 95 per cent. are killed in two hours.

Resistance to Drying and Antiseptics.—Simply drying destroys the majority of organisms dried at any one time, but some bacilli of the number dried may remain alive, especially when held in the texture of threads, for five or six months, or all may die in forty-eight hours.

They are killed in five or fifteen minutes in a 1 per cent. solution of carbolic acid.

Effect of Acids.—*B. communis* grows in a wider range of acids and alkalis than most other bacteria. It develops in from 0.2 to 0.4 per cent. of mineral acids, in from 0.3 to 0.45 per cent. of vegetable acids, and in from 0.1 to 0.2 per cent. of alkalis.

Effect of Intestinal Juices.—Gastric juice kills unprotected *B. communis* unless it is too greatly diluted by food. All the members of the typhoid-colon group are more resistant to the gastric juices than

[1] Jour. Hyg., 1905, **5**, 333.
[2] Centralbl. f. Bakt., 1895, **18**, 494.

most non-sporebearing bacteria. With the food they readily pass from the stomach into the intestines. They grow in bile and in the intestinal juices.

Pathogenesis.—*In Lower Animals.*—Intraperitoneal and intravenous inoculation of guinea-pigs and rabbits may produce death, which, when it occurs, usually takes place within the first forty-eight hours, accompanied by a decided fall of temperature, the symptoms of enteritis, diarrhea, etc., and finally fibrino-purulent peritonitis.

Subcutaneous inoculation into rabbits is followed usually by abscess formation at the point of inoculation. Dogs and cats are similarly affected.

Cystitis and pyelonephritis may be produced by direct injections into the bladder and ureters, if the urine is artificially suppressed. Angiocholitis and abscess are produced by direct injections into the liver. Osteomyelitis may follow the intravenous injections of cultures in young rabbits.

From experiments on animals it would appear that the explanation of the pathogenic effect of the colon bacillus is primarily due to an endocellular poison.

In Man.—In normal intestines with intact mucous membranes the toxic products formed by *B. coli* are absorbed but little or not at all, and the bacilli themselves are prevented from invading the tissues by the epithelial layer and the bactericidal properties of the body fluids. Possibly there is an acquired immunity to the colon varieties which have long inhabited the intestines.

B. coli was at first regarded purely as a parasite. Later, because of the postmortem invasion and the great ease of growth of the colon bacillus on ordinary media, the other extreme of attributing too much to it was taken.

The bacilli present in the intestines may, either by an increase in virulence or by a lowered resistance in the person, cause intestinal inflammation or more distant infections. Thus in the case of ulceration in typhoid fever *B. coli* may produce peritonitis. At times they pass through the intact mucous lining. The spread of bacilli from the intestines may cause disease in the gall-bladder or urinary tract. Agglutinins develop as a result of infection but this has not been used as a practical method of diagnosis as many serological varieties of B. coli exist. It is very difficult to determine in any colon infection whether the bacilli were previously present in the intestines or were derived from outside sources through water, food, or direct contact with other cases.

Virulence of B. Coli from Normal and Diseased Intestines.—The virulence varies with the culture and the time since its recovery from the intestines. Other things being equal, it is usually more virulent from an intestinal inflammation. From severe diarrhea the colon bacilli in 0.25 c.c. bouillon culture may kill guinea-pigs if given intraperitoneally, while from the healthy bowel 2.5 c.c. are usually required. The difference in man would probably be even greater.

B. Coli in Sepsis.—When lesions of the intestinal mucous membranes exist, or in B. coli cystitis, pyelitis or cholecystitis, there is frequently just before death a terminal dissemination of the bacilli and consequent septicemia. The colon bacillus septicemia is detected by blood cultures. At times very few bacilli are found, and then the blood infection may be less important than the local one. Secondary invasion of the blood stream has been observed in typhoid and cholera, especially in relapses in typhoid. In very young infants a malignant septicemia with tendency to hemorrhages may be due to *B. coli*. In a few cases in which *B. coli*, but no typhoid bacilli, were present the course of the disease has been similar to typhoid fever. An epidemic probably due to colon infection of water has been observed. Infections through food and water are usually caused by other closely allied bacilli not belonging to the colon group.

B. Coli in Diarrhea.—There is considerable question as to the significance of numerous epidemics which have been reported of acute diarrhea in children from one to five years of age in which almost pure cultures of colon bacilli have been found. Tissier has found that catharsis leads to an increase in the proportion of B. coli. It may be therefore, that such epidemics were due to another bacterium and that the predominance of B. coli was secondary to the diarrhea.

B. Coli in Peritonitis.—Not only perforation of the intestines in man, but injury to the intestinal walls, allows colon infection of the peritoneum to take place. At first most of these cases were believed to be due to a pure colon infection, but now it is known that this idea came largely from the overgrowth by colon bacilli of other bacteria. More careful investigations, through cultures and smears, have demonstrated the fact that streptococci, and less frequently staphylococci and pneumococci, are also usually present in peritonitis arising from intestinal sources. The colon bacilli found even in the same case commonly comprise many varieties.

B. Coli in Inflammation of the Bile Tract.—The normal healthy gall-bladder is usually sterile. This is true in spite of the fact that bile is apparently a good culture medium for the colon group. Ligation of the neck of the gall-bladder usually causes a colon infection to take place within twenty-four hours. Obstruction of the bile-duct inflammation is fairly common in man. The gall-bladder then becomes infected, and following the inflammation of the mucous membranes there is often the formation of gall-stones. Some cases of jaundice are believed to be due to colon inflammation of the gall-ducts. Atypical varieties of *B. coli* are frequently isolated from gall-bladder infections.

Inflammation of the Pancreas.—Welch was the first to record a case of pancreatitis with multiple fat necroses due to *B. coli* infection. A few more cases have since been reported due to members of the colon group, either alone or in conjunction with the pyogenic cocci.

Inflammation of the Urinary Tract.—As far back as 1879 Bouchard noted cystitis due to bacilli of the colon group. When cystitis is established the bacterial infection may spread to the pelvis of the kidneys,

causing a pyelitis or suppurative nephritis. From the pelvis the bacteria push up into the urinary tubules and excite inflammation and multiple abscesses. Colon infection of the different parts of the urinary tract may occur at any age, from infancy upward. Instead of starting in the bladder it may begin in the kidney itself, the colon bacilli coming from the blood or peritoneum.

Although other bacteria—the pyogenic cocci, the proteus bacillus, the typhoid bacillus, etc.—may excite cystitis, still in 90 per cent. of all cases some of the colon group are found, and this percentage is even higher in young children. The clinical picture of colon infection is very variable. The lightest cases progress under the guise of a bacteriuria. In the more severe cases there is an abundance of pus cells. The condition may last for weeks or months and then spontaneously disappear or grow worse. Pyelitis may develop, and this is usually indicated by an irregular intermittent fever resembling malaria. Septicemia may finally result.

In most of these cases the microscopic examination is sufficient to make a probable diagnosis, since the bacteria are so abundant. In the urine they appear as diplobacilli, or partly in short, almost coccus, forms, partly in long threads. As a rule motility is absent.

The characteristics of the urine itself have much to do with the probability of infection; the more acid urines being less likely to afford a proper soil for growth. Some urines are bactericidal even when they are neutral. The substances producing this condition are not known.

Retention of urine due to stricture or enlarged prostate are commonly predisposing causes of cystitis.

B. Coli as Pus Former.—Members of this group are frequently the cause of, or contributing cause of, abscesses in the region of the rectum, urethra, and kidney. They rarely produce pus in other locations.

B. Coli in Inflammation Not Previously Mentioned.—Broncho-pneumonia, lobar pneumonia, and pleurisy have occasionally been caused by colon bacilli, probably from blood sources. A few cases of meningitis and spinal meningitis in infants, follow localized *B. coli* infections. The symptoms are not well developed as a rule. Some cases of endocarditis and of conjunctivitis have also been noted.

Immunity.—Natural infection in man, or inoculation into animals, is followed by the production of antibodies; agglutinins, precipitins, bactericidal substances and opsonins being produced. The attempts to separate the members of this group by agglutination have shown the great dissimilarity of the different members in their immune reactions, although group reactions are marked. Natural agglutinins for this group are commonly present in the serum of man and animals. (See Agglutination.)

Vaccine Therapy.—Genito-urinary infections seem, in some instances, to be benefited, especially cystitis and possibly pyelitis after the acute symptoms have subsided. Vaccines may have some influence in diminishing the fever and discharge from sinuses after pelvic abscess, appendicitis or cholecystitis. The mucoid B. aërogenes (see below) as

well as intermediates resembling B. paratyphosus are frequently found in these types of infection. Bacterial vaccines of these types may be used if encountered. The dosage ranges from 25,000,000 to 500,000,000 or more. Different strains vary in the degree of reaction produced.

Methods of Isolation.—B. coli or its varieties may be isolated from lesions on ordinary media, in bile media or on Endo or Conradi plates. The Endo medium is used in isolation from feces or other mixed material. Blood cultures may be made in bile or broth. For the examination of water for members of the colon group, see Bacteriology of water.

Bacillus (Encapsulatus) Aërogenes and Allied Encapsulated Bacilli.—The members of this group differ from the members of the colon group, already described, in producing a more or less viscid or mucoid growth, and in smears a capsule is commonly demonstrable. For this reason the group is spoken of by some as the capsulatus group, and the aërogenes types described as an allied variety. There are wide variations in the group, not only in the degree of capsule production but in the ability to ferment different carbohydrates. In the absence of an accepted biological classification of types, the varieties are best considered according to their source. For this reason the term *B. aërogenes* is collectively used for the types normally found in feces, milk, etc.

B. Aërogenes.—Normally present in feces and also found in sewage and water. It is constantly present in milk and is one of the chief causes of the souring of milk and cream.

Morphologically it is variable in length and capsule production. In cultures the growth is abundant and may be viscid or mucoid in variable degree. It usually ferments the various carbohydrates more vigorously than other colon types, the closed arm of the fermentation tube being usually full or nearly full of gas. Indol production is variable. *B. aërogenes* is much more resistant to acids and other deleterious substances than the other colon types.

Pathogenesis.—It has been isolated from infections of the urinary tract, peritoneum and liver and gall-bladder. In the absence of reliable methods of differentiation there is always a question whether the more serious infections are not due to the more virulent bacillus of Friedländer.

Bacillus (Encapsulatus) Pneumoniæ, Friedländer (B. Mucosus Capsulatus).—This bacillus was first described by Friedländer in 1882. He confused this bacillus with the pneumococcus, then undescribed, and regarded it as the common causative agent in lobar pneumonia.

Morphology.—It varies from coccoid forms to longer bacilli which may be single, in pairs or in short chains. They are surrounded by a wide capsule which is easily demonstrable not only in the material directly from the lesions but also in smears from cultures.

Biology.—It grows freely on ordinary media. On agar the colonies are characteristically mucoid, with a tendency to confluence. Indol is not produced.

Pathogenesis.—It is pathogenic for rabbits, mice and guinea-pigs in variable degree. Injection intraperitoneally or into the lung is followed by peritonitis or local hepatization with septicemia.

In man it causes both broncho- and lobar pneumonia. This type of pneumonia is relatively infrequent and is characterized by its high mortality. The bacilli are present in the sputum, as a rule, in almost pure culture. Bacilli of the mucoid type are commonly present in the upper respiratory tract and the accessory sinuses, and may cause inflammation. By extension, infection of the ear occurs, which may be complicated by meningitis. The pleura or the pericardium may be infected as a complication of pneumonia. A septicemia may develop as a complication of pneumonia or infection elsewhere. Acute rhinitis and inflammations of the eye are occasionally due to this organism.

B. (Encapsulatus) Ozena.—This organism is culturally indistinguishable from the preceding. It receives its name from its constant presence in ozena or fetid rhinitis. Abel and others consider it the causative agent in this disease, basing their opinion on its constant presence.

Bacillus (Encapsulatus) Rhinoscleroma.—This bacillus differs from the *Bacillus pneumoniæ* only in its weaker fermentative properties. Its name comes from its frequent presence in rhinoscleroma. Its etiological relationship to this disease is not established. (See Fig. 14.)

Immunity and Serum Reactions of the Capsulatus Types.—Active immunity is difficult to produce in animals by the injection of killed cultures or their products. Careful injections of animals, however, lead to the production of agglutinins, precipitins, and complement-fixing antibodies. Different immunological types occur.

In common with other encapsulated bacteria, they may be resistant to action of antiserums. Porges found that the absence of agglutination could be attributed to the presence of the capsule and devised a method of destroying the capsule by first treating the bacterial suspension with acids and heat and then neutralizing with alkali.

Other Bacilli Allied to the Colon Group.—There are various types, of which *B. cloacæ* is an example, which resemble the colon bacillus in many respects but liquefy gelatin in varying degree, some very slightly and only after prolonged growth.

CHAPTER XXII.

THE TYPHOID BACILLUS.

(Genus Eberthella.)

This organism was first observed by Eberth, in 1880, in the spleen and disease areas of the intestine in typhoid cadavers, but was not obtained in pure culture or its principal biological features described until the researches of Gaffky in 1884. The absolute identification of the bacillus only became possible with the increase of our knowledge concerning the specific immune substances. Although an infection simulating typhoid fever cannot be produced in animals with the possible exception of the higher apes, there remains no question that B. typhosus is the etiological agent of typhoid fever. This has been proven by the development of specific immune substances in the blood of typhoid fever cases, by the constant finding of B. typhosus in the blood and in the lesions and most convincingly by laboratory accidents where swallowing of pure cultures of B. typhosus has caused typhoid fever and by the demonstration of the preventive value of B. typhosus vaccine.

Morphology and Staining.—Typhoid bacilli are short, rather plump rods of about 1μ to 3μ in length by 0.5μ to 0.8μ in diameter, having rounded ends, and often growing into long threads. They are longer and somewhat more slender in form than most of the members of the colon group of bacilli (Figs. 129 and 130).

The typhoid bacilli *stain* with the ordinary anilin colors, but a little less intensely than do most other bacteria. Like the bacilli of the colon and paratyphoid groups, they are decolorized by Gram's method. Bipolar staining is sometimes marked.

Biology.—The typhoid bacillus is a motile, aërobic, facultative anaërobic, non-liquefying bacillus. It develops best at 37° C.; above 40° and below 30° growth is retarded; at 20° it is still moderate; below 10° it almost ceases. It grows slightly more abundantly in the presence of oxygen. It does not form spores.

Resistance.—When a number of typhoid bacilli are dried most of them die within a few hours, the remainder gradually dying during the next few weeks. A few frequently remain alive for months. In their resistance to heat and cold they behave like the average non-spore-bearing bacilli. They are killed by heating to 60° C. in ten minutes, to 63° C. in five minutes.

Motility.—Typhoid bacilli, when living under favorable conditions, are very actively motile, the smaller ones having often an undulating

motion, while the larger rods move about rapidly. In different cultures, however, the degree of motility varies. Degraded variants are non-motile. (See Agglutination.)

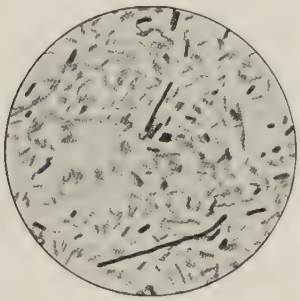


FIG. 129.—Typhoid bacilli from nutrient agar. × 1100 diameters.

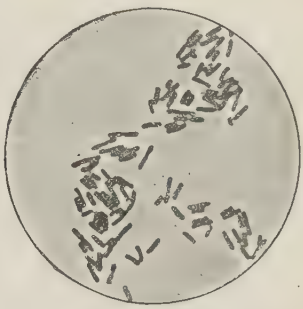


FIG. 130.—Typhoid bacilli from nutrient gelatin. × 1100 diameters.

Flagella.—These are often numerous and spring from the sides as well as the ends of the bacilli, but many short rods have but a single terminal flagellum (Figs. 131 and 132; see also Plate III).

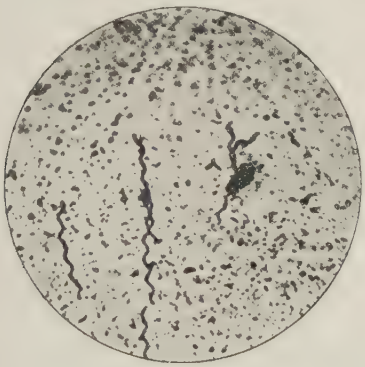


FIG. 131.—Flagella, heavily stained, attached to bacilli. (Van Ermengen's method.)

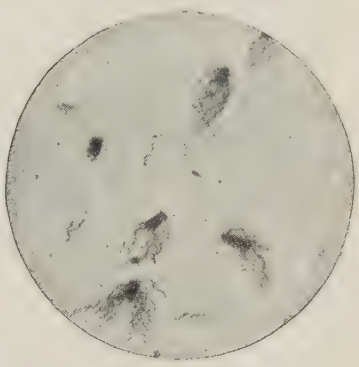


FIG. 132.—Typhoid bacillus with faintly stained flagella. (Löffler's method.)

Cultivation.—Its growth on most sugar-free culture media is quite similar to that of the *Bacillus coli*, but it is somewhat slower and not quite so luxuriant.

Growth on Gelatin Plates (Fig. 133).—The colonies growing deep down in this plate medium have nothing in their appearance to distinguish them from submerged colonies of the colon group; they are finely granular, with a sharp margin and a yellowish-brown color. The superficial colonies, however, particularly when young, are often quite characteristic; they are transparent, bluish-white in color, with an irregular outline, not unlike a grape leaf in shape. Slightly magnified they appear homogeneous in structure, but marked by a delicate network of furrows. Surface colonies from some varieties of colon bacilli give a similar picture.

In *stab cultures* in gelatin the growth is mostly on the surface, appearing as a thin, scalloped extension, which gradually reaches out to the sides of the tube. In the track of the needle there is but a limited growth, which may be granular or uniform in structure, and of a yellowish-brown color.

Growth in Bouillon.—This medium is uniformly clouded by the typhoid bacillus, but the clouding is not so intense as with the colon bacillus. The "rough" variant (see below) gives a granular growth which sediments. When the bouillon is somewhat alkaline a delicate pellicle is sometimes formed on the surface after eighteen to twenty-four hours' growth.

Growth on Agar.—The streak cultures on agar are not distinctive; a transparent, filiform, grayish streak is formed. Colonies on agar (and other solid media) may be of several kinds. The normal type is bluish-gray, moist and somewhat glistening. This is the "smooth" colony. A flatter matt irregular colony is common. This is the "rough" colony. Other forms are encountered, a compact small colony being common in old cultures, and this colony appearance is usually a definite sign of degradation.

FIG. 133.—A superficial colony and a deep colony of typhoid bacilli in gelatin. × 20 diameters.

Growth on Potato—The growth on this medium was formerly of great importance in identification, but now other media, giving more specific characteristics, have been discovered. When characteristic, the growth is almost invisible, but luxuriant, usually covering the surface of the medium. Again, the growth may be quite heavy and colored yellowish-brown, and with a greenish halo, when it is very similar to the growth of the colon bacillus. These differences of growth on potato appear to be chiefly due to variations in the substance of the potato, especially in its reaction. For the characteristic growth the potato should be slightly acid.

Indol Reaction.—It does not produce even a trace of indol in peptone-water solution. The typhoid bacillus, like the colon bacillus, produces alkaline substances from peptone.

Neutral Red.—In stab cultures in glucose agar or glucose broth cultures with neutral red as indicator the typhoid bacillus produces no change, while the colon bacillus decolorizes the medium and produces gas.

Effect of Inhibiting Substances in Culture Fluids.—The typhoid bacillus as a rule is inhibited by weaker solutions of disinfectants than is the colon bacillus. Some substances, such as brilliant green, inhibit the colon bacillus more.

Action on Different Sugars.—The essential fermentative difference between the typhoid bacillus and paratyphoid-colon group are given on pages 411, 414 and 440.

Milk.—The typhoid bacillus does not cause coagulation when grown in milk. A very slight acidity is evidenced in litmus milk from its action on traces of fermentable substances other than lactose.

Production of Disease in Animals.—It is impossible, with the exception of the anthropoid apes, to produce a disease like typhoid fever in animals. Metchnikoff and Besredka fed apes with typhoid bacilli by adding fecal material to their food. After eight days' incubation, fever, diarrhea and invasion of the blood stream by the typhoid bacilli, developed. The general clinical picture resembled that of typhoid fever. Some of the animals died. Injection of other animals may produce sickness due apparently to the toxic substances in the bodies of the bacilli injected. Typhoid bacilli, freshly obtained from typhoid cases and introduced into the peritoneal cavity may increase, causing a fatal peritonitis with toxic poisoning. By accustoming bacilli to the animal body a certain degree of increased virulence for the animal can be obtained, so that smaller amounts of culture may prove fatal. In rabbits a septicemia can be produced by intravenous inoculation. Localization in the gall-bladder follows in some and a "carrier" state similar to that found in man results. Direct injection into the gall-bladder also results in the development of the carrier state.

Distribution of Bacilli in the Human Subject. Toxic Effects.—Typhoid fever during its early stages, at least, is accompanied by a bacteriemia. The bacilli thus pass to all parts of the body and become localized in certain tissues, such as the bone-marrow, lymphatic tissues and spleen, liver and kidneys. Characteristic lesions develop in the intestine, especially in the lower part of the ileum, due to localization of B. typhosus in lymph nodules. Hyperplasia of the nodes with necrosis results in ulceration. Wherever found in the tissues the typhoid bacilli are usually observed to be arranged in groups or foci; only occasionally are they found singly.

Unusual Location of Typhoid Lesions Occurring as Complications of Typhoid Fever.—Cases of sacculated and general peritonitis, abscess of the liver and spleen, subphrenic abscess, osteomyelitis, periostitis, and inflammatory processes of other kinds have been reported as being due to the typhoid bacillus. In certain cases of typhoid pneumonia, serous pleurisy, empyema and inflammations of the brain and spinal cord on their membranes, typhoid bacilli exclusively have been found. The inflammation produced may or may not be accompanied by the formation of pus. There are indeed a number of cases now on record in which the typhoid bacillus has played the part of *pus producer*.

The Importance of Mixed Infection.—Frequently when complications occur in typhoid fever they are due to secondary or *mixed infection* with the staphylococcus, pneumococcus, streptococcus, pyocyaneus and colon bacillus. Frequently these bacteria are found side by side with typhoid bacilli; in such cases it is difficult to say which was the primary and which was the secondary cause of the complication.

Primary Infection of Liver and Gall-bladder.—We find an appreciable number of chronic typhoid carriers with no history of having had typhoid. Although the symptoms may have been so slight as to have been overlooked, it is probable that bacilli may escape from the intestine into the portal circulation and thus infect the liver and gall-bladder, the general immunity being sufficient to prevent the development of clinical signs of typhoid fever (see Normal Carriers).

Toxic Products of the Typhoid Bacillus.—It has not been determined what causes the toxemia of typhoid fever. It has been believed that this is largely due to the toxic bacterial proteins, "endotoxins." Toxic substances, which are heat-resistant, are demonstrable, however, in broth cultures. Schwartzman has recently reported a peculiar toxic effect in rabbits, a skin injection of broth filtrates followed twenty-four hours later by an intravenous injection of the same filtrate results in a severe hemorrhagic necrosis at the site of primary skin injection.[1]

Elimination of Typhoid Bacilli from Body.—Not infrequently typhoid bacilli are found in the secretions. They are present in the urine in about 25 to 50 per cent. of the cases during some parts of the disease, most commonly during the later periods of the disease.

Excretion is likely to be greatest during the third week of fever. They may be excreted, however, very early in the disease, and have been found in the stools even during the incubation period. Krumwiede, in a resurvey of convalescents in 1927, found that 25 per cent were excreting bacilli in their feces at about the twelfth day after normal temperature, 10 per cent. after about thirty days and 4 per cent. after about forty-five days. About 2 per cent. remain carriers.

Typhoid Carriers.—Petruschky, in 1898, reported that typhoid bacilli sometimes remained in the urine of typhoid convalescents for months. Cushing soon after observed a case who had had typhoid fever five years before. In 1902 Frosch, and a little later Conradi and Drigalski, reported persons who passed typhoid-infected feces months after recovery from typhoid fever. Accumulated evidence indicates that about 2 to 3 per cent. of convalescents become chronic carriers, some for the rest of their life.

As has been noted, carriers may be encountered who give no history of having had typhoid fever. In Washington, during the typhoid season, a series of 1000 examinations showed that 0.3 per cent. of persons with no known exposure, and who had not had typhoid were excreting typhoid bacilli. The carrier condition may arise after known contact or exposure and for this reason such carriers may be called "normal or contact carriers" in contradistinction to "convalescent carriers." The great majority of carriers are women. The incidence of carriers increases with age. The typhoid carrier is of great importance in the spread of infection (see Methods of Communication). The excretion by carriers is not necessarily constant. Periods of apparent intermittence occur and these may be prolonged.

[1] Jour. Exp. Med., 1928, **48**, 247.

The history of "Typhoid Mary," a cook, who was discovered by Soper, serves as an example of the possible dangers of a chronic carrier. Definite facts concerning her history could be obtained back to 1901. At this time a visitor of the family in which this woman was cook developed typhoid fever some ten days after entering the household. She went to another family. One month later the laundress in this family was taken ill.

In 1902 Mary obtained a new place as cook. Two weeks after arrival the laundress was taken ill with typhoid fever; in a week a second case developed and soon 7 members of the household were sick.

In 1904 she went to a home in Long Island. There were 4 in the family as well as 7 servants. Within three weeks after arrival 4 servants were attacked.

In 1906 she went to another family. Between August 27 and September 3, 6 out of its 11 inmates were attacked with typhoid. At this time she was first suspected. She entered another family on September 21. On October 5 the laundress developed typhoid fever.

In 1907 she entered a family in New York City, and two months after her arrival 2 cases developed, 1 of which proved fatal.

She was removed to a detention hospital March 19, 1907. Cultures taken every few days showed bacilli on and off for three years. Sometimes the stools contained enormous numbers of typhoid bacilli and again for days none would be found. She was released on parole in 1910, promising to report to the Health Department and not to engage in cooking. She broke her parole and disappeared. In 1915, in an epidemic of typhoid at a maternity hospital, a total of 25 cases developed. Investigation showed that food infection was the cause and the cook was identified as "Typhoid Mary." During the period of disappearance she infected a friend and was the cause of several cases in a small private sanatorium. Since then she has been again under detention.

The New York City Health Department and many others insist on at least two negative examinations of the feces of convalescents for B. typhosus before their discharge from quarantine. Those engaged in food-handling occupations are examined over a period of six months.

Krumwiede and Provost[1] have observed two chronic carriers who at times excreted only "degraded variants" in their feces. (See under Agglutination.) This should be considered where apparent B. typhosus colonies developing from the feces of carriers fail to agglutinate in a typical manner.

Treatment of Typhoid Carriers.—Meader believed that he obtained some success by vaccine treatment. We have been unable to obtain a cure by this method. Removal of the gall-bladder has been successful in some cases and not in others. Success would seem to depend on whether or not the chronic inflammation was limited wholly to the gall-bladder; however, there is a possibility that if the major reservoir,

[1] Unpublished. Mr. Mulcahy, of the New Jersey Health Department, sent the cultures and material from one, a known chronic carrier, because of the failure of the colonies to agglutinate.

the gall-bladder, is removed lesions of the bile capillaries will subside. All the apparent cures cannot be accepted as permanent, as a later recrudescence of latent lesions elsewhere in the biliary system is not excluded. Garbat's[1] conclusion that a safe indication of the absence of typhoid bacteria would be offered by two consecutive negative bile cultures and two consecutive negative feces cultures seems to us unwarranted. One of Nichols's[2] tables shows two negative duodenal cultures after cholecystectomy followed by a positive culture. Yet this case, it is stated, was discharged as cured after two further negative examinations. From this, it would seem that two or even three negative duodenal cultures are not acceptable as a criterion. As to fecal cultures, two or more may be negative. We have had negative fecal results with some chronic carriers over periods of one to three years. However, the evidence advanced indicates that a majority of carriers are cured by cholecystectomy.[3] It is unfortunate that not more of the operated cases were examined over a period of at least two or preferably three years.

The superiority of duodenal cultures as a criterion of the existence of the typhoid carrier condition has been emphasized. The advantages of obtaining material without a large associated flora, of avoiding the probability of the death of many of the typhoid bacilli during passage along the intestinal tract, cannot be denied. The reported differences in percentages between the two methods, we believe, would have been very much smaller had more adequate methods of fecal examination been employed.

Although cholecystectomy may result in a permanent cure, it is difficult to have carriers volunteer for this operation, especially when an absolute cure cannot be promised nor a complete absence of operative danger. The known typhoid carrier fortunately has proved to be a relatively small menace to the community or his or her family when under parole of the Health Department. The Health Department of New York City has well over a hundred carriers who are under parole. With exclusion from food-handling, instruction in personal hygiene, vaccination of members of the immediate family, etc., very few secondary cases develop. Krumwiede studied the histories of a series of carriers and calculated that at least 5 cases were due per carrier before the carrier condition was known; after discovery the rate is less than 1 case per 10 carriers. As the number before discovery is probably much too small, due to the lack of information, and the figure often too high, as no benefit of doubt was given, the actual differences are probably more striking.

The difficulty of control is the breaking of parole by the carrier and surreptitious handling of foodstuffs. This was the reason for a small outbreak due to ice-cream, which occurred recently in New York. If apprehended the only remedy is incarceration of the recalcitrant carrier.

[1] Rockefeller Institute Monographs, 1922, vol. **16.**
[2] Jour. Am. Med. Assn., 1917, **73**, 680.
[3] Haaland and Haaland: Lancet, 1927, ii, 1172.

Duration of Life in Water, Feces, Oysters, etc.—It is of importance to know for what length of time the typhoid bacillus is capable of living outside of the body; but, unfortunately, owing to the great difficulties in proving the presence of this organism in natural conditions, our knowledge on this point is still incomplete. In feces, in privies, on the ground, etc., the length of life of the typhoid bacilli is very variable, depending on the composition of the feces and the soil, on the temperature, and on the varieties of bacteria present; sometimes they live but a few hours, usually a day, exceptionally for very long periods. Thus, according to Levy and Kayser, in winter typhoid bacilli may remain alive in feces for five months. In contaminated oysters B. typhosus may remain viable as long as the oyster is alive and fit for consumption.[1] Their life in water is usually short, often not over forty-eight hours, and usually not over a week. With heavy contamination a few may persist longer. The less the general contamination of the water, the longer the bacilli are apt to live. The life of the typhoid bacillus varies according to the abundance and varieties of the bacteria associated with it, and according to the presence or absence of such injurious influences as deleterious chemicals, high temperature, light, etc., to which it is known to be sensitive. In ice typhoid bacilli rapidly die. Less than 1 per cent. remain alive after twenty-one days, after four months probably none survive.

Methods of Communication.—The bacilli may reach the mouth by means of infected fingers or articles of various kinds, or by the ingestion of infected food, milk, water, etc., or by more infrequent ways, such as the eating of raw oysters and clams, the use of contaminated ice or through the contamination of food by flies. Of great importance, though gradually lessening, however, is the production of infection by contaminated drinking water or milk. In a very large number of cases indirect proof of this mode of infection has been afforded by finding that the water had been contamined with urine or feces from a case of typhoid. In a very few instances the proof has been direct—namely, by finding typhoid bacilli in the water. Spring freshets are especially liable to wash fecal material into small streams. Wells are contaminated from nearby privies or through surface contamination with feces and urine.

The epidemic at Scranton, Pa., during the winter of 1907 was most interesting. A little over 1 per cent. of the inhabitants were attacked. Typhoid bacilli were isolated from the water of a small intercepting reservoir by Dr. Fox. This was only accomplished by using large quanties of water. The bacillus isolated was identical by all known tests with the typhoid cultures from cases of typhoid fever, except that it had very slight motility. A stream entering the reservoir was exposed to possible pollution by men working on a nearby railroad.

In water-borne outbreaks of typhoid fever it is not infrequent to have outbreaks of diarrhea preceding or accompanying the development of the typhoid fever. The number of cases of diarrhea may be many

[1] For fuller details, see chapter on Shellfish.

times greater than the number of typhoid cases. A sudden outbreak of diarrhea therefore may mean contamination of a water supply and should be considered as possible indication of an oncoming typhoid outbreak.

Milk infection may result from infected water. An example of this was seen in the case of a milk dealer whose son came home suffering from typhoid fever. The feces were thrown into a small stream which ran into a pond in which the milk cans were washed. A very alarming epidemic of typhoid developed, which was confined to the houses and asylums supplied with this milk. Milk-borne epidemics are most commonly due to infection of the milk by carriers. Food infection is likely to be source of infections limited to institutions or camps. In the latter flies may be contaminating agents, carrying the disease from open privies. Cases have been traced to oysters.[1] *Contact infection* is responsible for many cases. Even during outbreaks due to various causes, contact infection plays a large rôle in the development of secondary cases. These cases develop either by contact with known typhoid cases or through convalescent or healthy carriers in the households. The early undiagnosed cases may have typhoid bacilli in their stools and act as a source of contagion; likewise short, mild cases develop which are never diagnosed. For these reasons "typhoid precautions" should not await positive diagnosis, but should be instituted on the slightest suspicion. Contact with case or carrier is the major element in the persistence of typhoid fever ("residual typhoid") in cities where water and milk are no longer sources of infection.

Individual Susceptibility.—In this, as in all infectious diseases, *individual susceptibility* plays an important role in the production of infection. Where exposure is associated with the ingestion of few bacilli, the majority of persons escape infection. In many individuals there probably is some disturbance of the digestion, excesses in drinking, etc., or a general weakening of the power of resistance of the individual, caused by bad food, exposure to heat, overexertion, etc., as occurs with soldiers and prisoners, for example, to bring about the conditions suitable for the production of typhoid fever, when the dose of bacilli is small. With ingestion of larger numbers of bacilli and especially with repeated exposure, 50 per cent. or more of the exposed succumb to infection.

Immunity.—After recovery from typhoid fever a considerable immunity is present which lasts for years. This is apparently not absolute, as reports indicate that about 2 per cent. of those having typhoid fever have a second attack, which is usually a mild one. Some of these apparent recurrences however may have been paratyphoid fever. Specific *immunization* against experimental typhoid infection has been produced in animals by the usual method of injecting at first small quantities of the living or dead typhoid bacilli and gradually increasing the dose. The blood serum of animals thus immunized has been found to be highly bactericidal and to possess some protective power against

[1] See special chapter on Shellfish.

toxic extracts of the bacilli. It is also rich in agglutinins, precipitins and opsonins. These characteristics have also been observed in the blood serum of persons who are convalescent from typhoid fever. These parallel observations indicated to Pfeiffer and Kolle and to Wright (1896) that active immunization was feasible.

B. Typhosus Vaccine.—Prophylactic Use.—In 1898 Wright inoculated 400 men in India. Leishman supervised the injection of the British troops in the Boer War. In 1909 Russell started vaccination in the United States Army. The observations left no doubt as to the general efficacy of the procedure. The experiences in the World War were even more favorable. Vaccination against typhoid fever gives no appreciable protection against paratyphoid infection and combined vaccines should be employed wherever the latter is likely to be encountered.

The vaccine most generally employed is a saline suspension, heated to 53° C. from one hour to which 0.3 per cent. tricresol or carbolic acid is added to kill the residual living organisms.

Selection of Strains.—See Bacterial Vaccines for precautions, testing, etc.

Injections are given subcutaneously at weekly intervals, 500,000,000, 1,000,000,000 and 1,000,000,000. Smaller doses, but the same total, may be given if time permits, to lessen the risk of local or general reactions. Severe local reactions occur in a small percentage of vaccinated. Severe constitutional symptoms are less common. Several fatalities have been attributed directly to vaccination. However, the total persons vaccinated have been many millions. Exposure to infection is not a contraindication.

The degree of immunity given by typhoid vaccination is high and persists for an appreciable time. After five years the rate among the vaccinated will still be less than one-half that of the non-vaccinated. However, resistance is relative, depending on the degree of exposure and conditions of life. Our failures have been in doctors, nurses or those in immediate contact with carriers. Under conditions such as existed in the trenches during the World War revaccination in a year was found desirable.

Oral vaccination has been recommended by Besredka[1] and he believes that an immunity develops which will offer more protection than results from subcutaneous injection, because of the development of local tissue immunity or impermeability of the intestinal mucosa. The majority who have studied the use of oral vaccination in experimental animals[2] have observed an antibody response. Favorable reports have been made of the use in man, but more observation with greater controls are still needed before a final opinion can be given.

Lipovaccines, suspensions in oil, have been advocated and used. They are not satisfactory.

[1] Local Immunization, Williams & Wilkins, 1927.
[2] Burke and Barnes: Jour. Inf. Dis., 1926, **39**, 67.

Therapeutic Use.—The use by subcutaneous injection was summarized by Waters.[1] Of 1120 cases treated; 6.3 per cent. died. The incidence of relapse was 6 per cent. Some clinical betterment and apparent shortening of the disease was observed. Whether the lower death-rate was due to vaccination is a question. At least the results have not been sufficiently striking to induce the continuation of vaccine treatment.

The use intravenously[2] seems to have a definite tendency to abort the disease. A chill, a rise of temperature, and finally a sweating with fall in temperature usually occurs. The beneficial effects observed are probably due to protein shock or non-specific reaction. Fatalities due to intestinal hemorrhage, or to apparent embolism in patients with associated pneumonia led to fear of this mode of treatment.

The Laboratory Diagnostic Methods.—Four procedures are available: the blood culture, the urine culture, the feces culture and the Widal test. The following schematic outline indicates the relative value of these procedures in relation to period of the disease.

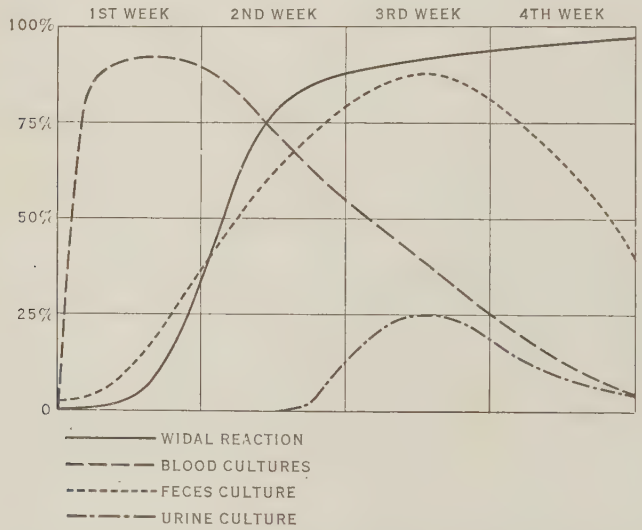


FIG. 134.—Approximate positive finding with different laboratory tests, according to week of disease in cases of about average duration.[3]

Diagnosis by Means of the Gruber-Widal or Agglutination Reaction.—The chief practical application of our knowledge of the specific substances developed in the blood of persons sick with typhoid fever has been as an aid to diagnosis. Widal published his data in 1896 and since then the test has come into general use. Although the earlier expectations have not been fully realized the method has been of great help as

[1] Med. Record, 1913, **84**, 518.

[2] Gay and Chickering: Arch. Int. Med., 1916, **17**, 303.

[3] The blood culture during the fourth week may be positive in about 25 per cent. of the very severe cases whose fever persists through the fifth week.

a diagnostic means or as a means to differentiate typhoid fever from similar febrile conditions. Its main drawback is the failure of the reaction in the early days of the disease.

Technic of Test.—See chapter on Agglutination.

Advantages and Disadvantages of Serum and Dried Blood for the Widal Test.—The dried blood is easily and quickly obtained, it is readily transported, and seems to retain the original agglutinating properties of the serum. It must in use, however, be diluted with at least ten times its original bulk with water, otherwise it is too viscid to be employed properly (see chapter on Agglutination).

Dilution of the Blood Serum to be Employed and Time Required for the Development of Reaction.—The results obtained in the Health Department Laboratories, as well as elsewhere, have shown that, employing the microscopic method, in a certain proportion of cases not typhoid fever a slow reaction occurs in a 1 to 10 dilution of serum or blood; but very rarely does a complete reaction occur in this dilution within *fifteen minutes*. From many cases examined it has been found that in dilutions of 1 to 20 a quick reaction is almost never produced in any febrile disease other than that due to typhoid or paratyphoid bacillus infection. In typhoid fever such a distinct reaction often occurs with dilutions of 1 to 100 or more. A complete reaction in a dilution of 1 to 20 in twenty minutes may be considered as positive. It is advisable to prepare a 1 to 40 dilution as a safeguard and as an additional index on the strength of the reaction. Cases of paratyphoid infection commonly give a prompt reaction in dilution of 1 to 20 or higher. As has been noted under agglutination titration of the agglutinin content may be employed to make a presumptive differential diagnosis between typhoid and paratyphoid fever. Typhoid-bacillus carriers with the exception of transient contact or normal carriers commonly have specific agglutinins in their blood whether they give a previous history of typhoid fever or not. Vaccination will also give rise to a positive reaction. These conditions must be considered in deciding on the diagnostic significance of a positive reaction.

The failure of the reaction in one examination by no means excludes the presence of typhoid infection. If the case clinically remains doubtful the examination should be repeated every few days. The incidence of the reaction is given in the tabular summary of diagnostic procedures.

Persistence of the Reaction after Convalescence or after Immunization.—A definite typhoid reaction has been observed from three months to a year after convalescence or after vaccination, and a slight reaction, though much less than sufficient to establish a diagnosis of typhoid infection, for a longer period. In persons in whom the typhoid bacilli persist the serum reaction may last as long as the bacilli remain in the body.

Diagnostic Value of Widal Reaction after Vaccination.—As agglutinins may persist for some time after vaccination one cannot be certain as to the interpretation to be placed on a positive reaction in the vaccinated. As already stated Dreyer believes that this difficulty may be overcome

by determining whether or not the agglutinins rise or fall, which change in a short period of time indicates an active infection. Blood culture is the better diagnostic method when cases are seen early.

The Widal Test in the Search for Carriers.—The indications are that the agglutinin content of the blood of carriers is subject to fluctuation and at any one time about 75 per cent. of chronic carriers will give a positive reaction. When using the Widal test in epidemiological work Krumwiede recommends the use of the regular dilutions and time of reading with a second reading at double the time. This will bring a greater number under suspicion for fecal examination and may locate a carrier which might otherwise be missed. He and others have obtained positive or highly suspicious reaction during apparent periods of intermittence of fecal excretion. The main advantage of the test is the possibility of a rapid survey of large groups. If nothing is found stools may have to be collected from all.

Typhoid Bacilli in the Blood.—A blood culture is usually positive during the first week of typhoid fever, and is the best method for early diagnosis. Bacilli appear in the blood even in the first few days of the disease. See Fig. 134 for percentage of positive findings. In relapses the bacilli reappear in the blood.

Method.—The blood is drawn from the median basilic vein by syringe, and is inoculated into either broth or bile medium. If broth is used, several flasks are used with sufficient broth to dilute the blood fifty times or more. The essential thing is to dilute the blood sufficiently so that coagulation does not occur. The separated serum apparently is more strongly bactericidal and inhibits growth.

Bile mediums are more convenient, as the bulk need not be so large. The bile inhibits coagulation. Three parts of bile medium are used to 1 part of blood. The bile is plated on Endo or Conradi, after eighteen hours' incubation. It should also be examined after two or three days, as growth is sometimes delayed.

Another method, not as satisfactory as the preceding but valuable in emergencies, is to inoculate the blood into ten times its volume of distilled water. Hemolysis results and the proteins supply the nutrient substances for growth. Where clotted blood is sent to the laboratory for the Widal test the clot may be cultured with a relatively high incidence of positive findings.

Typhoid Bacilli in Feces.—The more promptly the stools are plated after passage, the better is the chance of obtaining typhoid bacilli. Hiss, examining the stools very quickly after passage, obtained a relatively high percentage of positive results. See Fig. 134 for incidence of positive results. The failure to isolate B. typhosus from samples of feces which are over twelve to twenty-four hours old is due not only to the tendency of the bacilli to die but also to the overgrowth by associated bacteria. It has been recommended to emulsify 1 part of feces in 2 parts of 30 per cent. glycerin when a sample is likely to be delayed in transit to the laboratory. We have not found this method as valuable in practical work as we hoped it would be. Where there is likely to be any delay we prefer a sample of untreated feces as well, as this may be positive where the glycerin-treated specimens are negative.

Method.—The feces, if solid, are rubbed up with peptone-water, otherwise they can be used without further preparation. The fluidified feces should consist of a mixture of a generous amount of the whole mass, not merely a loopful.

The density of the suspension to employ is a matter of experience or successive dilutions may be plated. For general use the Endo or Conradi medium is most frequently employed. We employ Endo and a brilliant green agar (Krumwiede), using two concentrations of brilliant green (see Media), which by suppression of some or all of the fecal flora allows a heavy inoculation with coincident increase in positive results. None of the fluid enrichment media that we have tried have been satisfactory for general use, although they may be successful where the direct plating is negative. However, they frequently yield negative results where the direct plates are positive. In important cases enrichment in a correctly balanced range of brilliant green in broth should be tried, but plates should be made at least one, two, four and six days after inoculation if the maximum positive results are to be obtained.[1] Plates are streaked (see page 322) and the colonies of typhoid can then be agglutinated directly either microscopically or by using the slide method. In the latter a drop of saline for control and a drop of high-titer serum are placed on a slide and the colony rubbed up in the saline and then in the serum. With appropriate dilutions of the serum agglutination will take place almost immediately.

For fishing, Russell's medium, or, better, the Krumwiede triple sugar medium, is used, which gives a tentative indication of the nature of the organism, which can be confirmed by agglutination and further cultural tests.

Typhoid Bacilli in the Urine.—The results of the examination of others as well as our own indicate that the typhoid bacilli are not apt to be found in the urine until the end of the second week of the fever, and may not appear until much later. From this on to convalescence they appear in about 25 per cent. of the cases, usually in pure culture and in enormous numbers, even as high as 1,000,000 per cubic centimeter. They may be found until several weeks or months after convalescence; in exceptional cases they persist for years. Persistent urinary carriers are very much less frequent than are fecal carriers.

Method.—If the bacilli are numerous, as evidenced by examination in the hanging drop, they are easily obtained by direct plating, otherwise the urine should be centrifuged and the sediment plated, and larger amounts inoculated into bile medium for enrichment.

Typhoid Bacilli in Rose Spots and Spleen.—Although the bacilli have been frequently isolated from rose spots, it is a less convenient method than blood cultures. Spleen puncture has been employed and although cultures are usually positive the operation is dangerous, and has been abandoned.

Detection of Typhoid Bacilli in Water, Milk, Oysters, Etc.—There is absolutely no doubt that the contamination of streams and reservoirs is a frequent cause of the outbreak of epidemics of typhoid fever, but the actual finding and isolation of the bacilli is rarely successful. Some of the reported positive findings are in doubt today. The difficulty in examining water, oysters, etc., is that by the time examination is undertaken the contamination may be past. It is very difficult to find

[1] For example, see Krumwiede, Park, Cooper, *et al.*: Am. Jour. Pub. Health, 1926, **14**, 263.

a very few bacilli among numerous and usually more vigorous associated bacteria. This difficulty is greatest with milk. Presumptive indication of fecal contamination is usually obtained in an indirect way by testing for B. coli. (See Water, etc.)

Identification of B. Typhosus.—As bacilli resembling B. typhosus in all morphological and cultural respects are encountered, identification rests on serological reactions. The typhoid bacillus is easily separated from the other members of the group, and for practical purposes it is sufficient identification if the colony on the special plating media and the growth on Russell's medium or its modifications, is characteristic and is agglutinated in relatively high dilutions of a serum, so as to eliminate the action of group agglutinins. If a strain does not agglutinate freely, as commonly happens with strains isolated from the blood, it should be transferred daily on plain agar and again tested.

In important examinations or strains from unusual sources as water, etc., a more complete cultural and serological study should be done.

CHAPTER XXIII.

PARATYPHOID-ENTERITIDIS GROUP.

(Genus Salmonella.)

Gärtner, in 1888, found a bacillus in association with a meat-infection epidemic. He named it Bacillus enteritidis. A cow sick with diarrhea had been slaughtered and the meat sold for food. Of the persons eating the meat 57 became ill.

Bacilli similar in many respects were isolated by Smith from swine suffering from hog cholera and from mice by Löffler. Paratyphoid bacilli were also isolated by others from human cases resembling typhoid fever. Schottmüller reported in 1900 that he was able to differentiate paratyphoid strains into two groups, one differing slightly from B. typhosus and the other considerably. The work of Durham and Buxton gave the basis for a further classification into subgroups.

Numerous types have been isolated from infections in man and animals, and most of the terms in use refer to their source. Gas production from glucose is usually given as the differential characteristic between the paratyphoid-enteritidis group of bacilli and B. typhosus. This characteristic is susceptible to variation and may be lost. From our own work it would appear that the ability to ferment rhamnose is a more basic characteristic of the paratyphoid-enteritidis group. The more the strain resembles the typhoid bacillus, however (especially the non-gas-producing type mentioned above), the lower is the avidity for this carbohydrate. The ability to ferment salicin or saccharose serves to exclude many of the paratyphoid-like organisms found in feces. Indol also is not produced by the types in this group known to be pathogenic for man.

Paratyphoid A. (Salmonella paratyphi).—Disease Produced.—It was first isolated by Gwyn. It produces a typhoid-like disease in man, but is of relatively infrequent occurrence in temperate areas but more frequently encountered in warmer areas. The bacillus may be present in the feces, urine, blood and bile. The postmortem findings have been variable. In a few the typical intestinal lesions of typhoid infections were present, in others they were absent. A diffuse inflammation of the intestines may be found.

Morphology and Biology.—It is similar to typhoid in many respects. Ford and also Krumwiede[1] have shown that it differs from all other members of the paratyphoid-enteritidis group in that it does not produce acid from xylose. Not all the non-xylose-fermenting types from man are alike agglutinatively and the latter observer suggests that all be included in the paratyphoid "A" group and that the strains differing agglutinatively be considered a subgroup. This fermentative reaction is more

[1] Jour. Med. Res., 1916, **24**, 335.

reliable than the differentiation by litmus milk. The reaction on litmus milk has, since Schottmüller's work, been relied upon for differentiation of B. paratyphosus A from other members of the paratyphoid group. The reaction, however, is only quantitative and exceptions occur.[1]

Communicability.—Communicability is the same as for typhoid.

Diagnosis.—See below.

Paratyphoid B. (Salmonella Schottmülleri).—This type is primarily a human pathogen different from the similar paratyphoid types found in animals. Paratyphoid fever due to B. paratyphosus "B" is transferred from man to man directly or indirectly as in the preceding enteric fevers. The acute infections or intoxications resulting from meat infection are usually due to other varieties of paratyphoid bacillus. In a few instances food infection has been attributed to the true "B" variety.

Disease Produced in Man.—Clinically it usually resembles typhoid fever. As a rule, the bacteriemic character of the disease is more marked than with B. typhosus infections.

Morphology and Biology.—They closely resemble the other members of this group, but grow more freely and rapidly than B. typhosus. For differential characteristics, see pp. 410 and 411, and large table, opposite p. 151.

Occurrence in Healthy Persons; Paratyphoid Bacillus Carriers ("A" and "B" Types).—When general invasion occurs the bacilli may be found in the urine after convalescence for shorter or longer periods of time. Its localization in the gall-bladder and its excretion in the feces by chronic carriers is fairly frequent. We[2] had the opportunity of examining the feces of every man in a militia regiment which had been badly infected with paratyphoid "A," while at the Mexican border. We found nearly 4 per cent. of healthy carriers. This incidence of normal carriers is interesting in relation to normal carriers of B. typhosus. Is this greater incidence only apparent, due to the greater ease with which we can isolate B. paratyphosus as compared with B. typhosus or actual, because, the former known to be less virulent, a greater number of persons escape infection but become carriers for a shorter or longer time? Careful bacteriological examination has revealed paratyphoid-like bacilli in the feces of a considerable percentage of healthy persons. This fact is unfortunately lost sight of, and etiological importance ascribed to such organisms when isolated from the feces during disease. Because of the range of group agglutination, such organisms may even be agglutinated by the patients' serum.

Other Paratyphoid Varieties in Paratyphoid Fever.—Varieties which are closely allied to, if not identical with the types to be described under food infection have been encountered in some cases of paratyphoid fever. In some instances the disease was in epidemic form. In the majority of these instances the causative bacterium belonged either to the B. pestis caviæ group or to the B. suipestifer group. The paratyphoid C, Hirschfeld, the Glasser-Vodagsen strains, etc., belong in this group.

[1] Krumwiede, Pratt and Kohn: Jour. Med. Res., 1916, **34**, 355.
[2] Krumwiede: Jour. Inf. Dis., 1917, **21**, 141.

Frequency of Paratyphoid Infections.—In this country in the Northeast at least, the disease is relatively infrequent, though statistics are not available. It is frequent in Europe, especially in certain districts. In the World War the disease was very common. In this country military camps have been favorable places for its development as seen in the outbreak of paratyphoid ("A") fever in the militia at the Mexican border.

Communicability.—The individual case and the carrier either through contact or by the contamination of water and milk, etc., are the usual sources of infection for paratyphoid fever. In the case of other varieties it is possible that infection may arise from animal sources.

Diagnosis of Paratyphoid Fever.—As with typhoid fever the blood culture is the diagnostic method of choice. A positive blood culture is likely to continue longer than in typhoid fever. The blood culture method is the surest means of making a differential diagnosis, especially if the patient has been vaccinated. The isolation from the urine is also valuable. The presence of bacilli in the feces is not necessarily diagnostic as this may be due to the carrier condition. Differential diagnosis by agglutination gives us only presumptive evidence. (See Agglutination.) The difficulties of drawing a conclusion are greatly enhanced when vaccination has been carried out. As has been previously noted, Dreyer believes that these difficulties may be overcome by determining the rise and fall in agglutinins for one of the types, that is, for B. typhosus and B. paratyphosus "A" or "B". Agglutinins develop later and usually less freely in infections due to B. paratyphosus "A". Where isolation methods are employed the isolated type must be identified, not only culturally (see below), but also by agglutination with known type sera, and in the case of the more closely related types by use of the agglutinin-absorption method. The methods of isolation given for B. typhosus are directly applicable for the paratyphoid types. The latter are more resistant to brilliant green so that their isolation from feces is relatively easy.

Immunity and Vaccination.—Immunization has been very successful during the World War, a triple vaccine containing B. typhosus and B. paratyphosus "A" and "B" being employed. A vaccine containing B. paratyphosus A and B. paratyphosus B, 750,000,000 per cubic centimeter of each, may be employed. More commonly the so-called triple vaccine is employed. This has the above content of B. paratyphosus plus 1,000,000,000 of B. typhosus per cubic centimeter. One-half, 1 and 1 c.c. doses are usually given at weekly intervals. Smaller and more frequent injections may be given to avoid severe reactions, giving, however, the same total as above. Oral vaccination has been recommended. Löffler as early as 1906 showed that the feeding of mice would result in immunity. Besredka believes that this method will be at least as effective as subcutaneous injection. Vaccination is specific. Protection against other types would require vaccines of those types.

Food Infection ("Poisoning") Due to Paratyphoid Types.— Quite a number of paratyphoid varieties have been found to be the causative agent in food infection. Three paratyphoid varieties, however, have

been the most frequent causative agents, members of the B. pestis caviæ (termed B. aërtrycke by some), B. suipestifer and B. enteritidis groups. The food infection is manifested by the development of an acute gastro-enteritis. The period of incubation may vary from a few hours to one or two days. The length of the period of incubation depends apparently on the number of bacteria ingested, that is, on the degree of contamination of the foodstuff and on the amount eaten. The suitability of the food as a culture medium for the bacterium and time and temperature of storage are evidently factors. The bacilli may invade the blood stream and may find their way to the urine. This is most likely to be so in fatal cases.

These bacteria have the ability of producing toxic products and the idea that these preformed toxic products cause the gastro-enteric symptoms has been very generally accepted. The opinion is gaining ground that the disease is primarily an infection.

The paratyphoid varieties encountered in food infection are primarily animal pathogens and commonly cause infection of food-producing animals which infections usually terminate in a septicemia. As would be expected, therefore, meat or meat-containing foods have been common sources of infection. The meat from a healthy animal may be contaminated by contact with meat of a diseased animal. Cooking may fail to kill all the bacteria in the interior of a piece of meat. The handling of contaminated meat may lead to the contamination of other food. We[1] encountered an example of this where tapioca pudding was apparently contaminated with B. suipestifer from pork. Another source of probable contamination is rodents. We[2] recently encountered an example of this danger. An epidemic was found to be due to cornstarch-cream filled cakes contaminated with B. pestis caviæ. Investigation showed that the filler was undoubtedly contaminated from rodent droppings. Rodent contamination may have been the source of infection in some other epidemics, such as that reported by Winslow.[3]

Diagnosis.—Diagnosis depends on the isolation and identification by cultural and agglutination reaction of the offending type from the food or from the infected individual. The proof that the bacilli from both sources are identical completes the chain of evidence. A positive agglutination reaction with the patient's serum will usually develop about a week to ten days after infection. Diagnosis by this means is rendered difficult because of the marked group agglutination among many of the food infection varieties.

Other Forms of Infection Due to Paratyphoid or Paratyphoid-like Bacilli.[4]—Local infection of the biliary system or of the genito-urinary tract may

[1] Krumwiede, Cooper and Provost: Jour. Med. Res., 1921, **43**, 55.
[2] Salthe and Krumwiede Am. Jour, Hyg., 1924, **4**, 23.
[3] Am. Jour. Hyg., 1923, **3**, 238.
[4] See Krumwiede, Kohn and Valentine: Jour. Med. Res., 1918, **38**, 89; 1919, **39**, 449, for details and references; also Ten Broeck: Jour. Exp. Med., 1918, **28**, 759; Schutze: Lancet, 1920, **1**, 93; Jordan: Jour. Inf. Dis., 1923, **33**, 567 and Salthe and Krumwiede: Am. Jour. Hyg., 1924, **4**, 23. Andrews and Neave: Brit. Jour. Exp. Path., 1921, **2**, 157. Jordan: Am. Jour. Pub. Health, 1927, **17**, 1221.

complicate paratyphoid fever or may develop as primary infections. In the latter instance one is likely to encounter paratyphoid-like varieties which are serologically different from the recognized paratyphoid types. Occasionally, paratyphoid-like varieties are encountered in terminal septicemia.

SUMMARY OF THE COMMONER MEMBERS OF THE PARATYPHOID-ENTERITIDIS GROUP.[1]

B. paratyphosus A and B. paratyphosus B.—Commonest causative agents in paratyphoid fever in man.

B. pestis caviæ (*B. aërtrycke, Mutton Type*).—Common causative agent in food infection, encountered also in enteric fever. Probably it is primarily an animal pathogen, of wide species pathogenicity[2] commonly encountered in rodents, many of the so-called typhi-murium cultures belong in this group, also encountered in ducks,[2] swine (hog cholera), calves, rabbits, cats and canaries. B. psitticosis belongs in this group.

B. suipestifer or B. choleræ suis Group.—Commonly encountered in food infection. It has also been found in continued fevers of enteric type. These cultures have been designated as *B, paratyphosus C* in many instances. The Hirchfeld and Glasser-Voldagsen strains fall into this group. (See also below under Miscellaneous.) Common secondary invader in hog cholera. Some of the cultures labelled B. icteroides fall into this group.

B. enteritidis.—Common agent of food infection. Encountered in infections of food-producing animals. Common in rodents and some so-called typhi-murium cultures belong in this group. This and B. pestis caviæ have been employed in the commercial viruses used for eradicating rodents.

B. sanguinarium.—So-called fowl typhoid bacillus.

B. pullorum.—Causative agent of white diarrhea in chicks.

B. anatum.—Infections in ducks, some of the cultures under this name belong to the B. pestis caviæ group.

B. abortus equi.—Contagious abortion of mares.

Miscellaneous.—Varieties closely allied to the above varieties especially to B. paratyphosus B. and B. pestis caviæ are encountered occasionally in food infections and in continued fevers. The term B. paratyphoid C. has been employed by some to designate various miscellaneous varieties. When adequately studied some of the cultures so designated will be found to fall in the above groups. The term as used therefore designated a heterogeneous group and is not desirable. As was noted paratyphoid-like bacilli or intermediates are common.

Cultural Reactions.—Considerable confusion has existed as to the fermentative capacities of the members of this group. This was largely due to the failure to appreciate the differences in avidity exhibitable by different cultures of the same group or by strains from the same culture. This variability is emphasized in the following table. It should be emphasized that agglutination and in some instances agglutinin absorption is necessary for final identification.

Agglutination Reactions.—Group agglutination is common. It is usually very marked among cultures of B. paratyphosus B. and B. pestis caviæ and certain miscellaneous varieties which culturally resemble these. Agglutinin absorption may be necessary for a final decision as to the identity of a culture. The above varieties also show cross agglutination with the B. suipestifer varieties, the degree varying with different serums. Here likewise agglutinin absorption may be necessary. B. typhosus and B. enteritidis varieties may show considerable cross agglutination. The cultural differences and titration of the serum will usually serve for differentiation. B. typhosus, B. pullorum and B. sangui-

[1] If Salmonella is the genus accepted all of these belong to this genus.

[2] Some strains termed B. anatum belong here.

narium show very marked group reactions. The difficulties of identification may be materially increased by the common occurrence of agglutinative variants, group and specific components, etc. (see under Agglutination).

MAIN DIFFERENTIAL CULTURAL CHARACTERISTICS.[4]

Groups or types.	Glucose serum water.[2]	Lead acetate agar.	Gas from glucose.	Acid Production from.[3]					
				Rhamnose.	Maltose.	Xylose.	Arabinose.	Dulcit.	Inosite.
B. typhosus	+	+(± or −)	−	−	+	+ or ±	− or ±	± or −	−
B. sanguinarium	irreg.	irreg.	−	±	+	+ or ±	+	+ or ±	−
B. pullorum	"	"	+(−)[1]	+	−	+ or ±	+	−	−
B. paratyphosus "A"	+	−	+	+	+	−	+	±	−
B. abortus equi.	+	−	+	+	+	+	+	+	−
B. chol. suis	+	−	+(−)[1]	+	+	+	− to ±	− to +	−
B. paratyphosis "B"	−	+	+(−)[1]	+	+	+	+	+	+ or −[2]
B. pestis caviæ	−	+	+	+	+	+	+	+	+ or −[2]
B. enteritidis	−	+	+	+	+	+	+	+	−

Symbols: Glucose serum water, + = pink, no reduction.
− = pale or nearly colorless reduction.
Lead acetate agar, + = browning, sulphide production.
− = no change.
Fermentation, + = prompt, ± delayed, − negative.

Notes: 1. Exceptional findings.
2. Strains showing these differences may be identical agglutinatively and on agglutinin absorption. All freshly isolated "B" types have fermented this substance.
3. All produce acid from glucose, galactose, levulose and mannite. None produce acid from lactose, saccharose, raffinose, erythrite, adonite, salicin or inulin.

Media: Carbohydrates, 1 per cent. in peptone water, Andrade indicator.
Glucose Serum Water: Horse serum, 1 part; distilled water, 4 parts, glucose, 0.1 per cent.; Andrade indicator, 1 per cent. Steam twenty minutes for two successive days.
Lead Acetate Agar: Cool agar (in bath) to 50° to 60° C., add sufficient 0.25 per cent. aqueous solution of basic lead acetate to give 0.05 per cent. concentration; inoculate by stab between agar and glass. Test suitability with known strains.

4. Based on studies of group by Krumwiede, Kohn, Cooper, Rosenstein, etc., in this laboratory.

Colony and Serological Variants.—Colony variants are commonly encountered in this group. They may appear on plating media seeded directly with material from the infected host. What may be considered the normal colony is smooth. Rough, compact or opaque and mucoid colony variants as well as other colony forms are encountered. Antigenic change may be observed with all types, though the compact colony seems always to be degraded. Virulence is generally low with the rough, though not invariably so. Krumwiede and Cooper have observed rough variants of B. suipestifer which were fully virulent for rabbits and the rough variant was recovered at death. Group and specific components may be found in individual cultures. (See under Agglutination.) This phenomenon is not paralleled by differences in colony morphology.

CHAPTER XXIV.

DYSENTERY GROUP (GENUS EBERTHELLA).

Dysentery may be divided into acute and chronic. Amebæ appear to be the chief exciting factor in most cases of chronic dysentery, though bacilli of the colon group also play a part.

In temperate climates acute dysentery is but very rarely due to amebæ, but usually to the bacilli identified by Shiga or to allied strains later identified by Kruse, Flexner, Park, Hiss and others. The usual summer diarrheas are not excited by the dysentery bacilli.

Historical Note.—In 1897 Shiga found in the stools of cases of dysentery a bacillus which had not been before identified. In 1900 Flexner and Strong isolated bacilli which they at that time considered the same as those isolated by Shiga. In the same year Kruse, in Germany, isolated bacilli from cases of asylum dysentery which in their agglutinative characters were not all alike. Park and Dunham (1902) isolated a bacillus from a severe case of dysentery during an epidemic at Seal Harbor, Mt. Desert, Maine, which they showed differed from the Shiga dysentery bacillus in that it produced indol and differed in agglutinating characteristics. At first they considered it the same as the Flexner strain, but it was shown later by Park to be a distinct variety, and later found by him in widely separated epidemics.

Martini and Lentz, in December, 1902, found that the Shiga type was present in separate epidemics in Europe, but also in some cases, that other types similar to those isolated by Flexner, Park, Kruse, and others were found. These types differed from the Shiga type in fermenting mannite and in agglutination. In January, 1903, Hiss and Russel showed that a strain isolated by them differed from the Shiga type in the same characteristics as the Park-Dunham culture.

German observers at first were inclined to consider the Shiga type as the only one producing dysentery, while the American observers considered the other varieties to be causative agents. Park investigated several epidemics and isolated only the Shiga type from some, from others either the Park-Hiss or the Flexner types, thus definitely proving the importance of the mannite fermenting types. The results obtained by others later were the same, so that no doubt exists that the paradysentery varieties cause true dysentery. Park was the first to apply the name Paradysentery to these forms.

Morphological and Cultural Characteristics of Dysentery Bacilli (Placed in the Genus Eberthella by S. A. B.).—Microscopic.—Similar to bacilli of the colon group.

Staining.—Similar to bacilli of the colon group.

Motility.—Although non-motile, molecular movement is very marked. Flagella are absent.

Appearance of Cultures.—On *gelatin* the colonies appear more like the typhoid than the colon bacilli. Gelatin is not liquefied. On agar growth is somewhat more delicate than that of the average colon cultures. Both "smooth" and "rough" colony types occur. The latter is usually degraded. (See Agglutination.)

On Potato.—A delicate growth just visible or distinctly brownish.

In Bouillon.—Diffuse cloudiness with slight deposit and sometimes a pellicle.

See page 411 for comparison with other members of colon-typhoid group.

Varieties of Dysentery Bacilli.—B. dysenteriæ and the paradysentery varieties are generally accepted to be causative agents in clinical dysentery. Other somewhat similar bacilli have been encountered in dysentery cases where the above types were not isolated. Etiological significance has been attributed to these varieties but this has not obtained any general agreement. Of these B. ambiguus seems most likely to have some connection with clinical dysentery. The fermentation reactions used for the separation of the dysentery bacilli and the above noted related varieties are given in the following.

	Glucose.	Mannite.	Lactose.	Dulcite.	Indol.
B. dysenteriæ Shiga	+	−	−	−	−
B. paradysenteriæ, Park-Hiss, Flexner, etc.	+	+	−	−	+
B. ambiguus, B. dysenteriæ Schmitz	+	−	−	−	+
"Dulcite fermenters" B. alkaligenes	+	+	−	+	+
"Lactose fermenters" B. dispar Kruse E	+	+	+	−	+

There is a marked tendency among B. dysenteriæ to vary in their carbohydrate fermentation. This is more marked with some carbohydrates than with others. The reactions given above are the more stable ones. Subdivisions of the paradysentery group based on the fermentation of maltose and saccharose are frequently given; thus that the Park-Hiss type ferments neither maltose nor saccharose and that the Flexner variety ferments maltose but not saccharose. Absolute reliance cannot be placed on such fermentative reactions. As an example Sonne found the following apparent groupings according to the time of incubation of the cultures inoculated in carbohydrate media.

Twenty-four hours —Park-Hiss 42; Flexner 32 } Total,
Forty-eight hours —Park-Hiss 31; Flexner 43 } 74 strains.

The fermentation reactions will vary also in successive tests at considerable intervals. If other carbohydrates are added, other apparent subgroups appear. These fermentative differences may show little correlation with antigentic differences.

At least three definite agglutinative varieties of dysentery bacilli were demonstrated by the work of Park, Lentz, Hiss and others. B. dysenteriæ Shiga is sharply differentiated from the paradysentery varieties. The Park-Hiss and the Flexner varieties, however, although

they show marked group reaction can usually be separated by resort to absorption.

Sonne has studied a large series of cultures from sporadic cases; and based on agglutination suggests the following classification.

Group I.—*Park-Hiss types* (this group will include some cultures giving the fermentative reaction usually attributed to the Flexner type).

Group II.—*Flexner* (includes the so-called Strong type; some cultures may have fermentative characteristics usually attributed to the Park-Hiss types).

Group III.—*Sonne* (includes types with so-called Flexner fermentative characteristics).

He isolated a few strains which apparently did not fall into the above groups.

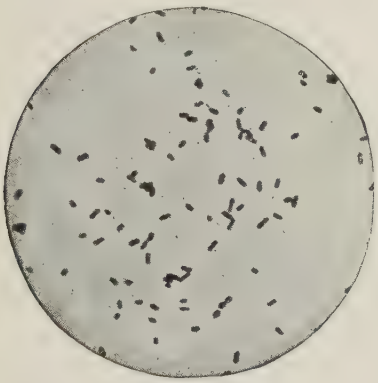


Fig. 135.—Dysentery bacilli. × 1000 diameters.

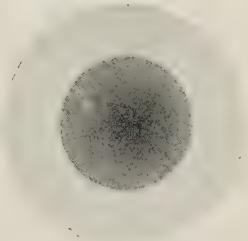


Fig. 136.—Colony of dysentery bacilli in gelatin. × 40 diameters.

During the war there were many dysentery cases in the English armies. The cultures obtained were studied by Murray,[1] Andrews and Inman[2] and others. Stock cultures obtained from different laboratories were included for comparison. Andrews and Inman subdivide the para-dysentery varieties into groups which they designate as V, W, X, Y and Z, and intermediate groups VZ and WZ. The letters designate what they believe to be the major "antigenic constituents" of the bacilli. They found that the cultures obtained from different laboratories as Park-Hiss or as Flexner, for instance, fell into more than two groups. We believe that their group V corresponds to the Flexner type and the Y to the Park-Hiss type, as they contain the Lentz type cultures which agreed with those originally studied by Park, Hiss and others. Whether their other larger group Z corresponds to the Sonne type remains to be determined.

Although the Park-Hiss and Flexner types are probably the more

[1] Jour. Roy. Army Med. Corps, 1918, **31**, 257 and 353.
[2] Nat. Health Ins.; Med. Res. Committee Special Report Series, 1919, vol. **42**.

commonly occurring varieties, especially in this country, the recent investigations indicate that other serological varieties exist although some are only differentiable by very close study of their serological characteristics.

The main importance of these findings is the indication that the selection of cultures for the production of antiserums for diagnostic or therapeutic purposes, or for diagnostic agglutination reactions should be based on a study of the locally occurring varieties and the necessity of selecting dominant types. (See under Agglutination.)

Pathogenesis. — Animal Tests. — No characteristic lesions with one exception have followed the feeding of large quantities of bacilli. Dogs at times have diarrhea with slimy stools, but autopsy shows merely a hyperemia of the small intestine. The disease can be produced and occurs spontaneously in monkeys. Many animals are very sensitive to dead or living bacilli injected into the subcutaneous tissues, vein or peritoneal cavity.

The autopsy of animals dying quickly from injection into the peritoneum of living or dead bacilli shows the peritoneum to be hyperemic, the cavity more or less filled with serous or bloody serous exudate. The spleen is sometimes moderately swollen. The small intestine is filled with fluid, the large intestine is usually empty The mucous membrane of both is hyperemic and sometimes shows hemorrhages.

Subcutaneous injections of dead or living cultures are followed by infiltration of tissues and frequently by abscess formation. The dysentery bacilli are not found in the blood or organs of animals.

Toxins.—Poisonous products, "endotoxins," are found in autolysates and in filtered broth cultures. When injected into animals death results and on autopsy the same lesions are found as following the injection of live or dead bacilli. This endotoxin can be neutralized by large doses of immune serum. Kraus and others have demonstrated that the Shiga type also produces a true extracellular toxin against which an antitoxin can be produced. This explains why the Shiga type is the more toxic, the others less so or irregularly so.

The transmissible lytic substances of the dysentery bacilli are considered under the general discussions of this subject (p. 68).

In Man.—The etiological significance of the dysentery bacillus in man is not only shown by its constant presence and by the presence of immune bodies in the serum of infected persons, but also by experimental infection of man. Thus Strong infected two condemned criminals with pure cultures. Jehle infected himself. Kruse reported two accidental laboratory infections, and others have occurred.

As a general rule infection with the Shiga type causes a more severe disease and the mortality is higher than in infection from other types. The severity of individual cases, however, varies widely during an epidemic.

Prevalence of the Disease.—The disease is distributed over the whole world. The Shiga type and Park-Hiss type of the paradysentery varieties are most commonly found in the United States.

Distribution of the Bacilli.—The bacilli are only found in the intestines. They do not invade the rest of the body. The feces, therefore, are the only excretions containing them.

Duration of Life Outside of the Body.—They usually die in stools in one to two days. In water they die out in several days to a week, exceptionally after a longer time.

Communicability.—The infected person is mainly responsible for the spread of dysentery. Especially dangerous are the mild or subacute cases or carriers. Such infection may be direct or it may be indirect through contamination of food, dishes, linen and clothes; water may also be infected and be the cause of epidemics. Milk has been the source of infection in a number of instances. The bacillus has been isolated from water during epidemics.

Bacillus Carriers.—Both healthy and convalescent carriers are found. The former may be equal to one-fourth or even one-half of the number of cases. Convalescents commonly excrete the bacilli for weeks and some become chronic carriers. The excretion is irregular, and slight relapses occur, at which time the bacilli are more numerous. The carrier condition seems wholly due to the persistence of chronic lesions of the intestinal mucosa. The importance of finding and isolating the carriers during an epidemic is obvious. This is not always possible and general precautions against infection should be taken.

Susceptibility.—The frequent occurrence of healthy carriers and mild cases along with severe and fatal cases shows the varying resistance to infection. Disturbances of digestion and other conditions lowering the general resistance, such as heat and fatigue, are factors in infection.

Immunity.—Immune bodies appear in the blood some time after infection. In animals, immune bodies are also produced by injection. Agglutinins, bactericidal substances, precipitins and opsonins are found. Neutralization of the toxic products is also possible with immune sera. A true antitoxin is also probably produced for the Shiga type.

Active immunity in animals is produced with difficulty against the Shiga type because of its high toxicity. Against the other types, however, immunity is more easily produced.

Dysentery Vaccines.—The prophylactic injection of dysentery bacillus vaccines seems to lower the morbidity and mortality. There is one serious difficulty, the marked toxicity of such vaccines. Shiga attempted to meet this difficulty by simultaneous injection of vaccine and antiserum. Many investigators have studied the possibility of oral vaccination. Besredka has recently centered attention on this procedure. A few reports[1] have been made on oral vaccination in man and they seem favorable.

Serum Therapy.—Antibacterial serums having a high antibody content can be prepared with B. dysenteriæ (Shiga); the serum will also be antitoxic. Polyvalent antiserums are usually prepared. The serum is most efficacious in the Shiga types of dysentery. The presumption is that the curative effect is largely antitoxic in character. Fifty to 80 c.c.

[1] Enslow: Pub. Health Rept., 1925, **40**, 639.

of serum is given, preferably intravenously, once or twice a day for the first few days of the disease.

Diagnosis.—The use of the agglutinating reaction with the patient's serum is limited, because the symptoms are sufficiently diagnostic in well-marked cases and, further, the reaction does not appear during the early acute stage. Later it may be used. Group or normal agglutinins are commonly present and interfere greatly with the value of the reaction in the paradysentery types. The differences in agglutination of B. dysenteriæ and the paradysentery group is usually sharp, but infection by the types of the paradysentery group can frequently not be differentiated. An agglutination reaction is commonly present in chronic carriers. For even moderate diagnostic value the serum should agglutinate in 1 to 50 in infections with *B. dysenteriæ* and in 1 to 100 with other types.

Isolation of the bacillus is the only method of diagnosis for certain identification of the type causing the infection. The mucous flakes in stools should be selected for plating. The methods of isolation and identification are the same as for typhoid or paratyphoid, except that the crystal violet should be omitted from the Conradi medium as the growth of many strains is inhibited more or less by anilin dyes. In acute cases the bacilli are so abundant in the mucus of most cases that they can be readily isolated from nutrient agar plates. The isolation from carriers is not always easy. If Endo is used the plates should be fresh and the reaction not too alkaline. A large content of fuchsin or the presence of unreduced fuchsin will inhibit the growth of B. dysenteriæ. Krumwiede[1] showed that fuchsin almost acted as a specific inhibiting agent with the Shiga type. Veal infusion on a peptic digest basis seems to be more desirable for this group.

Differential Diagnosis of Type.—The cultural differences have been given. The actual nature of the bacillus should be verified by agglutination. The cultural characters have only tentative value.

Agglutination.—Sera from rabbits or goats are used, as horses develop normal and group agglutinins to a high degree.

For practical purposes two sera, one for B. dysenteriæ and a polyvalent serum for the paradysentery types may be used. For differentiation of the subgroup monovalent sera are necessary. Park-Hiss and Flexner sera will contain considerable group agglutinins for each other and for other paradysentery varieties. It is desirable that serum for the Sonne type may be included to determine whether strain types occur in this country. As dysentery strains vary widely in their agglutinability failure of identification due to relative inagglutinability should be followed by repetitions of the test. If necessary absorption may be resorted to.

As we have shown certain anaërogenic colon-like organisms are occasionally encountered which are agglutinated in high dilutions of dysentery sera. These may be excluded if they possess microscopic or cultural characteristics known not to be possessed by the dysentery bacillus. If necessary agglutinin absorption may be resorted to.

[1] Jour. Exp. Med., 1914, **19**, 20.

CHAPTER XXV.

BACILLUS PYOCYANEUS (BACILLUS OF GREEN AND OF BLUE PUS). BACILLUS PROTEUS (VULGARIS).

The two genera described in this chapter are found often in the alimentary canal. Usually they are commensals that show pathogenicity only under certain conditions lowering the hosts susceptibility. They are not classified together. Pyocyaneous is placed with the genus Pseudomonas in the first tribe (chromobacterieæ) of the family Bacteriaceæ, and Proteus is a genus of Tribe VII (Bacteriaceæ) of this family.

BACILLUS PYOCYANEUS (PSEUDOMONAS ÆRUGINOSA).

The blue and green coloration which is occasionally found to accompany the purulent discharges from open wounds is usually due to the action of the *Bacillus pyocyaneus*. It was first obtained in pure culture and its significance noted by Gessard, 1882.

Morphology.—Slender rods from 0.3μ to 1μ broad and from 2μ to 6μ long; frequently united in pairs or in chains of four to six elements; occasionally growing out into long filaments and twisted spirals. The bacillus is actively motile, a single flagellum being attached to one end. Does not form spores. *Stains* with the ordinary anilin colors; does not stain with Gram's stain. Stained with alkaline methylene blue it may show polar granules and look a little like a small diphtheria bacillus.

Biology. — Aërobic, facultative anaërobic, liquefying, motile bacillus. Growing anaërobically it produces no pigment. Grows readily on all artificial culture media at room temperature, though best at 37° C., and gives to some of them a bright green color in the presence of oxygen. In *gelatin-plate* cultures the colonies are rapidly developed, imparting to the medium a fluorescent green color; liquefaction begins at the end of two or three days, and by the fifth day the gelatin is usually entirely liquefied. The deep colonies, before liquefaction sets in, appear as round, granular masses with scalloped margins, having a yellowish-green color; the surface colonies have a darker green center, surrounded by a delicate, radiating zone. In *stab cultures in gelatin*, liquefaction occurs at first near the surface,

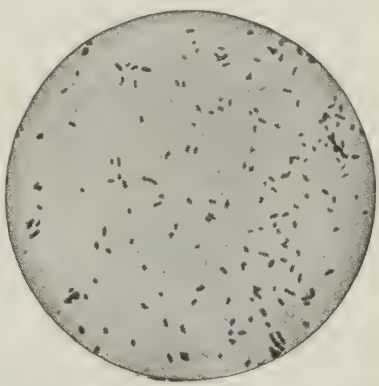


Fig. 137. — Bacillus pyocyaneus. (From Kolle and Wassermann.)

in the form of a small funnel and gradually extends downward; later the liquefied gelatin is separated from the solid part of the medium by a horizontal plane, a greenish-yellow color being imparted to that portion which is in contact with the air. On *agar* a wrinkled, moist, greenish-white layer is developed, while the surrounding medium is bright green; this subsequently becomes darker in color, changing to blue green or almost black. In *bouillon* the green color is produced, and the growth appears as a delicate, flocculent sediment. *Milk* is coagulated and assumes a yellowish-green color.

Pigment.—Two pigments are produced by this bacillus—one of a fluorescent green which is common to many bacteria. This is soluble in water but not in chloroform. The other (pyocyanin) of a blue color is soluble in chloroform, and may be obtained from pure solution in long, blue needles. This pigment distinguishes the *Bacillus pyocyaneus* from other fluorescing bacteria. Pigment production is usually more marked with incubation at 22° C. The ability to produce pigment may be lessened or lost by artificial cultivation.

Ferments.—Besides the ferment causing liquefaction of gelatin there is one which acts on albumin. It resists heat. This ferment called pyocyanase is able to dissolve bacteria, and it has been stated to have some protective power when injected into animals. It has been used locally in diphtheria in a number of cases. We think it has no advantage over cleansing preparations.

Both an endotoxin and an exotoxin have been described for this bacillus but little recent work has been done on these poisons.

Several investigators have described the formation by this organism of a transmissible lytic substance.[1]

Distribution.—This bacillus is very widely distributed in nature, it is frequently found on the healthy skin of man, in the feces of many animals, in water contaminated by animal or human material, in purulent discharges and in serous wound secretions.

Pathogenesis.—Its pathogenic effects on animals have been carefully studied, especially on guinea-pigs and rabbits. Subcutaneous or intraperitoneal injections of 1 c.c. or more of a bouillon culture usually cause the death of the animal in from twenty-four to thirty-six hours. Subcutaneous inoculations produce an extensive inflammatory edema and purulent infiltration of the tissues; a serofibrinous or purulent peritonitis is induced by the introduction of the bacillus into the peritoneal cavity. The bacilli multiply in the body and may be found in the serous or purulent fluid in the subcutaneous tissues or abdominal cavity, as well as in the blood and various organs. When smaller quantities are injected subcutaneously the animal usually recovers, only a local inflammatory reaction being set up (abscess), and the animal is subsequently immune against a second inoculation with doses which would prove fatal to an unprotected animal.

Its presence in wounds in man greatly delays the process of repair,

[1] Hadley: Jour. Inf. Dis., 1924, **34**, 260.

and may give rise to a general depression of the vital powers from the absorption of its toxic products. This bacillus has been obtained in pure culture from pus derived from the tympanic cavity in disease of the middle ear, from cases of ophthalmia, and bronchopneumonia. Kruse and Pasquale have found the organism in three cases of idiopathic abscess of the liver, in two of them in immense numbers and in pure culture. Ernst and Schürmayer report the presence of the bacillus pyocyaneus in serous inflammations of the pericardial sac and of the knee-joint. Ehlers gives the history of a disease in two sisters who were attacked simultaneously with fever, albuminuria, and paralysis. It was thought that they would prove to have typhoid fever or meningitis, but on the twelfth day there was an eruption of blisters, from the contents of which the Bacillus pyocyaneus was isolated. Krambals refers to seven cases in which a general pyocyaneus infection occurred, and adds an eighth from his own experience. In this the Bacillus pyocyaneus was obtained postmortem from green pus in the pleural cavity, from serum in the pericardial sac, and from the spleen in pure culture. Schimmelbusch states that a physician injected 0.5 c.c. of sterilized (by heat) culture into his forearm. As a result of this injection, after a few hours he had a slight chill, followed by fever, which at the end of twelve hours reached 38.8° C.; an erysipelatous-like swelling of the forearm occurred, and the glands in the axilla were swollen and painful. Wassermann reports an epidemic of septic infection of the newborn, starting in the umbilicus. In all there were eleven deaths. Lartigau found it in well water, and in great abundance in the intestinal discharges of a number of cases made ill by drinking the water. It has also been found in a certain number of cases of gastro-enteritis in which no special cause of infection could be noted.

We may therefore conclude from these facts that the Bacillus pyocyaneus, although ordinarily but slightly pathogenic for man, may under certain conditions, as in general debility, become a dangerous source of infection. Children would seem to be particularly susceptible.

Differentiation of B. Pyocyaneus from Other Fluorescing Bacteria.—This is easy enough as long as it retains its pigment-producing property. When an agar culture is agitated with chloroform a blue coloration demonstrates the presence of this bacillus. When the pyocyanin is no longer formed, however, the differentiation is by no means easy, particularly when the pathogenic properties are also gone.

Immunity.—Animal infection is followed by the production of antitoxic and bactericidal substances.

PROTEUS VULGARIS.

The term Proteus vulgaris is used for a group of bacilli, types of which were first isolated by Hauser in 1885 from putrefying meat. The limits of this group are not well defined. Wenner and Rettger[1] have

[1] Jour. Bact., 1919, 4, 331.

reported a systematic study of the proteus group in which they show that several strains classed as different varieties are probably identical and that other strains included in this group do not belong in it. They subdivide the group on the basis of their action on maltose into two species, namely, proteus vulgaris, fermenting the sugar and proteus mirabilis failing to attack it. The genus cannot be subdivided satisfactorily on the basis of proteolytic action, indol production or agglutinating properties. The following is a description of proteus vulgaris.

Morphology.—Bacilli varying greatly in size; most commonly occurring 0.6μ broad and 1.2μ long, but shorter and longer forms may also be seen, even growing out into flexible filaments which are sometimes more or less wavy or twisted like braids of hair.

The bacillus does not form spores, and *stains* readily with fuchsin or gentian violet. It is Gram-negative.

Biology.—An aërobic, facultative anaërobic, liquefying, motile bacillus, which ferments glucose, sucrose and maltose with the production of acid and gas. Grows rapidly in the usual culture media at room temperature.

Growth on Gelatin.—The growth upon *gelatin plates* containing 5 per cent. of gelatin is very characteristic. At the end of ten to twelve hours at room temperature small round depressions in the gelatin are observed which contain liquefied gelatin and a whitish mass consisting of bacilli in the center. Under a low-power lens these depressions are seen to be surrounded by a radiating zone composed of two or more layers, outside of which is a zone of a single layer, from which ameba-like processes extend upon the surface of the gelatin. These processes are constantly undergoing changes in their form and position. The young colonies deep down in the gelatin are somewhat more compact, and rounded or hump-backed; later they are covered with soft down; then they form irregular, radiating masses, and simulate the superficial colonies. When the consistency of the medium is more solid, as in 10 per cent. gelatin the liquefaction and migration of surface colonies are more or less retarded. In *gelatin-stick* cultures liquefaction takes place rapidly along the line of puncture, and soon the entire contents of the tube are liquefied.

Growth on Nutrient Agar.—A rapidly spreading, moist, thin, grayish-white layer appears, and migration of the colonies also occurs. *Milk* is coagulated, with the production of acid and the later digestion of the casein.

Cultures in media containing albumin or gelatin have a disagreeable, putrefactive odor, and become alkaline in reaction. Growth is most luxuriant at a temperature of 24° C., but is plentiful also at 37° C. It is an aërobic bacillus but it grows also in the absence of oxygen. In the latter condition it loses its power of liquefying gelatin. It produces indol and phenol from peptone solutions. Proteus vulgaris develops fairly well in urine, and decomposes urea into carbonate of ammonia.

Pathogenesis.—This bacillus is pathogenic for rabbits and guinea-pigs when injected in large quantities into the circulation, the abdominal cavity or subcutaneously, producing death with symptoms of poisoning. Hauser has obtained the Proteus vulgaris from a case of purulent peritonitis, from purulent puerperal endometritis and from a phlegmonous inflammation of the hand.

It is probable that some instances of enteritis have been due to the contamination of foods by Proteus. Because of the proteolytic power, toxic products "ptomaines" may develop as a result of its growth. Under these conditions decomposition has started and the food is disagreeable both in taste and odor, and for this reason food "poisoning" of this type is probably uncommon.

Proteus vulgaris has been found to be the predominating organism in the alvine discharge in cases of cholera infantum. The prominent symptoms in these cases were drowsiness, stupor and great reduction in flesh, more or less collapse, frequent vomiting and purging, with watery and generally offensive stools.

The Proteus vulgaris appears to be next in importance to the Colon group in the etiology of cystitis and pyelonephritis.

The Proteus vulgaris is usually a harmless parasite when located on the mucous membrane of the nasal cavities. Here it only decomposes the secretions, with the production of a putrefactive odor. It is found occasionally in the discharge from cases of otitis media in association with other bacteria.

For the use of certain proteus strains in the diagnosis of typhus fever see that disease.

CHAPTER XXVI.

ALKALIGENES—BRUCELLA GROUP.

Castellani and Chalmers (1919) described as a new genus "those Bacteriaceæ growing well on ordinary laboratory media; not forming endospores; aërobes, and often facultative anaërobes; without fluorescence, pigment formation or gelatin liquefaction; without polar staining; Gram-negative, without a capsule. . . . Milk not clotted; glucose and lactose not fermented." They gave it the name Alkaligenes. According to this definition the bacillus of Malta fever and that causing contagious abortion—B. abortus of Bang would fall into this group—but Evans[1] has pointed out that there are enough points of diffeerenc between the alkaligenes forms, so similar to the typyoid bacillus and the melitensis-abortus forms, that were long thought to be cocci, to consider them separate genera. Chief among these points are the much smaller size and coccoid forms of the latter and their power to invade animal tissues. She therefore recommends that the name Brucella proposed by Meyer and Shaw (1920) be adopted for such a genus. The species bronchisepticus also falls into this Brucella group. This leaves for the Alkaligenes group only a few presumably non-pathogenic forms similar to Alkaligenes fecalis, found in the intestinal canal and in milk.

ALKALIGENES FECALIS.

This bacillus resembles the bacillus typhosus in morphology staining reactions, motility and in the general appearance of its growth on or in media. However, it differs in its inability to ferment any carbohydrates. In milk progressive alkalinity occurs. The bacillus is markedly aërobic in growth. It would be of little interest, other than as a parasite of the intestinal tract, but for its reported isolation from a few cases of continued fever resembling a mild typhoid fever.

THE BRUCELLA GROUP.

In this group is included the causative organism of Malta fever, termed in the past Micrococcus melitensis (Bruce), the B. abortus (Bang) type, the causative agent of contagious abortion in cattle and the B. abortus of infectious abortion in swine (Good and Smith). The terms caprine, bovine and porcine are employed to designate the commonest natural host origins and the apparent host adaptations of the members of the Brucella group. The first is the Brucella melitensis,

[1] Pub. Health Rept., U. S. Pub. Health Serv., 1923, **38**, 1943.

the second and third, Br. abortus. The causative agent of a Malta fever-like symptom complex in man commonly called undulant fever is Br. abortus. This is discussed below.

Morphology.—The strains of different host origins are not distinguishable by morphological examination. The dominant form is the short ovoid bacillary form so closely approximating a coccus that Bruce applied the term micrococcus. This coccoid form varies from 0.3μ to 0.5μ in its longer diameter. More definite rod forms of 1μ to 2μ are seen in smear. They stain comparatively faintly as a whole. Occasional individuals take the stains more deeply or show a metachromatic granule. They are decolorized by Gram's method. Neither spores, capsules nor flagellæ have been seen. The organism is non-motile.

Biology.—These organisms grow feebly on or in ordinary agar and broth. Minute dew drop colonies develop on agar, usually taking two to three days to become macroscopically visible. In broth there is a slight turbidity. Growth is better on liver-digest agar or with the addition of serum or ascitic fluid. Directly from the infected host, there is a distinct microaërophilic tendency. This is especially marked with the bovine variety. Shake cultures in serum-agar reveal this characteristic by the location of the growth close to the surface. In the isolation of the bovine variety the inoculated plates are incubated in a sealed container, with B. subtilis cultures. It was assumed that success was due to oxygen absorption to the appropriate point for growth. One square centimeter of B. subtilis culture to 15 c.c. of volume of air is the best proportion. Another procedure is to seal the inoculated tubes. Growth will appear very slowly. This also has been attributed to the absorption of oxygen. Smith's observations show that the production of CO_2 is the more determining factor in these isolation methods. Prompt growth occurs if CO_2 is present. Thus if 10 per cent. of CO_2 is present (mixed with N or H) apparently mere traces of oxygen will suffice for growth. The porcine and caprine strains may be isolated without such extra precautions. On further cultivation the apparent microaërophic character is lessened so that the bovine strains grow with free circulation of air, likewise growth is better with all strains than at the time of isolation. Indeed, they may continue growing slowly for several days until the mass growth appears comparatively dense. The optimum temperature is 37° C. A moderate alkalinity of media is best. No carbohydrates are fermented, gelatin is not liquefied and milk becomes alkaline.

Resistance.—The members of this group are more resistant than their relatively feeble growth on media would indicate. They are killed by heating to 140° F. for ten minutes, at 142° F. for seven and a half minutes and at 145° F. for five minutes.

Pathogenicity.—The natural hosts of the Brucella group are the goat, the cow and the swine. Sheep may also be infected. In the goat infection may be manifested by abortion or by mastitis. The microorganism is excreted in the urine and milk. In cows and swine infection results in abortion. Milk from many of the infected cows contains the organism. Infection may be artificially induced by the races

from heterologous species, but apparently the homologous strains seem to have developed a more or less definite species pathogenicity. Guinea-pigs may be infected. This has occurred spontaneously through contact infection. The abortion strains have been studied in relationship to their pathogenicity in guinea-pigs. Several observers have stated that the porcine strain is more virulent than is the bovine strain. Large doses will kill acutely. With moderate to small doses with resulting subacute infection there develops an enlargement of the lymph nodes and of the spleen. If inoculation were by the intraperitoneal route peritoneal adhesions may be present. The enlargement of the spleen may be so marked that the organ seems fairly bursting. With chronic disease the enlargement is only moderate and the organ flabby. The surface may be normal or present numerous fine pin-point elevations. Tiny gray foci may be distinguished just beneath the capsule. On section the pulp is usually soft and swollen but distinct foci are seldom seen. The liver may show a few to many grayish foci, barely visible or up to 2 to 3 mm. in diameter. Grayish foci may be seen in the kidneys. Similar foci may be observed in the lungs just beneath the pleura. The testicles, bones, joints, very infrequently the uterus, may show lesions. Microscopically the foci of infection show epithelioid proliferation and microscopic examination shows that the foci are more numerous and more general in the organs than macroscopic appearances suggest. This is due to the fact that only the very advanced lesions are visible to the eye. The tissue changes closely resemble those of tuberculosis. Pregnant guinea-pigs may abort. Comparing the picture obtained in guinea-pigs injected with strains of bovine, porcine and human origin (undulant fever) Smith[1] found consistent differences. The bovine strains caused endothelial proliferative lesions which might be 0.4 to 0.6 mm. in diameter and necrosis was usually absent. With the porcine strains, some or all of the foci enlarge until they become visible as yellow masses 2 to 3 mm. in diameter. Sections show extensive polymorph infiltration with or without necrosis, the extent of this invasion of the endothelial foci and the necrosis running parallel with the size of the foci. The strains of human origin gave a similar picture. As strains in cultivation may lose their virulence such differences may only appear with freshly isolated cultures. A similar investigation of the caprine is needed before the comparative characteristics of the group can be clearly established. In man the disease produced is Malta fever or commonly called undulant fever where caprine sources of infection are not present. The disease is characterized by irregular and intermittent fever. The infection may be very prolonged. That the goat is a source of infection to man is definitely determined, the source of infection being usually milk. Foci of Malta fever of caprine origin were recognized in the southwestern states over twenty years ago. It is only in the last few years, however, that it has been recognized that an undulant fever was fairly common in this country (and in

[1] Smith: Jour. Exp. Med., 1926, **43**, 207.

other countries) which had no possible caprine source. When it became evident that a very considerable amount of cows' milk was infected with B. abortus the question whether man was susceptible to infection naturally arose. Investigations showed that agglutinins could be demonstrated in the blood which raised a presumption of possible infection. However, it was not until January, 1924, that conclusive evidence was advanced by Keefer who reported a case of apparent Malta fever. He obtained Br. abortus from the blood. The cultural and serological tests on the strains from further cases of human infection leaves no question that the etiological microörganism in undulant fever is a Brucella type. With the knowledge that from 10 to 30 per cent. of milch herds are infected, that cows' milk commonly contains B. abortus, it was natural to assume that this was the usual source of infection where milk from other species was excluded. Contact with swine was not ascertainable except in isolated cases, such as that of Blake, whose patient worked in slaughter house and handled swine. Reconsideration of this obvious conclusion seems necessitated by the following facts. Although the total number of recognized cases is growing to considerable numbers the number is still not proportionate to the probable incidence of B. abortus in cows' milk. Fabyan injected monkeys with B. abortus (bovine) without effect. Fleischner, Vecki, Shaw and Meyer concluded from their experiments that the bovine may be pathogenic for monkeys but that B. melitensis was much more invasive. Huddleston[1] finds that large doses of the bovine type are necessary to cause a mild and transient infection in monkeys, whereas the swine type in nearly every case where one agar slant of culture was fed produced the disease in ten to fifteen days without recovery. Small doses of a human strain of recent origin were also virulent but less so than those of direct swine origin. Nicolle injected 5 human beings subcutaneously with 800,000,000 to 900,000,000 of the bovine type without evident effect. Infection of laboratory workers has been frequent indicating that the strains virulent for man have a high pathogenicity. Finally we have the contrast in guinea-pigs between the bovine type and the porcine type, and the likeness of lesions produced by the porcine type and strains of human origin as described by Smith. Smith believes that "we must be prepared to look for the source of human infections in the bacillus of swine abortion provided the caprine type is not in evidence." This conclusion does not mean that cows' milk is not the immediate source of infection for it appears to be in most instances. Infection of cattle, possibly at times only, udder invasion by the swine type presumably must occur to explain cows' milk transmission and this infection must pass to other cattle if infection in man is due to the porcine type as seems indicated by the data given. The number of cultures, thus far studied in the above regards is not great. The questions are; will further study show constant contrasts and likenesses, are there occasional bovine strains of greater virulence for man, have all or few porcine strains potential infec-

[1] Jour. Bact., 1927, **17**, 58.

tiousness for cattle? In France infection in man has also been attributed to sheeps' milk. Whether there is any species adaptation in sheep remains to be studied. Monkeys are easily and certainly infected by the caprine variety.

Diagnosis of Infection in Man.—Three means are available, blood and urine cultures and agglutination. Blood or urine cultures are commonly positive at the height of the fever. Agglutinins appear in the blood after the infection has existed for a week or longer. A positive reaction in a dilution of 1 to 50 raises a presumption of past or present infection. A positive reaction in 1 to 100 gives a practical certainty, which certainty becomes greater with positive reactions in higher dilutions. The reaction may be observed with the microscopic or macroscopic method. We prefer the latter method using a freshly prepared saline suspension from young agar culture. The suspension should have only a slight turbidity. Incubate for two hours at 50° to 55° C. The microscopic method is trying on the eyes because of the small size of the bacterium.

Serological Varieties.—There is a very close serological likeness among the great majority of the members of the Brucella group without regard to their origin. Simple agglutination tests will not serve to separate the cultures into groups. Agglutinin absorption however, will show differences. These differences do not correlate with host origins. Evans[1] has studied a considerable series of cultures. She found that the strains of Mediterranean origin (caprine and human) fell mostly into three groups which groups differed from most of the strains obtained elsewhere. Nearly all of the latter strains fell into two groups.

BRUCELLA BRONCHISEPTICUS.

In 1910 Ferry,[2] reported the isolation of a small, Gram-negative, slightly motile bacillus from the bronchi in a canine distemper. McGowan (1911) found the same organism. It grows slowly and delicately, most typically on potato. It ferments no sugars. Ferry and Noble[3] have given differential points between it and Klebsiella ozenæ. The recent report of Laidlow and Dunkin[4] seems definitely to prove what has been contended by others, that distemper is primarily due to a filtrable virus. The ferret is susceptible to the virus. Vaccines made from the tissues of infected animals give a high degree of resistance. Bacillus of guinea-pig pneumonia Theobald Smith (1914) showed was the same as Br. bronchisepticus.

[1] Hygienic Labr. Bull., 1925, No. 143.
[2] Am. Vet. Rev., 1910, p. 499.
[3] Jour. Bact., 1918, **3**, 499.
[4] Brit. Med. Jour., 1928, ii, 100.

CHAPTER XXVII.

MICROÖRGANISMS BELONGING TO THE HEMORRHAGIC SEPTICEMIA GROUP.

(Genus Pasteurella.)

A number of bacilli of similar characteristics have been described as causing certain infectious diseases of lower animals, marked by the appearance of hemorrhagic areas throughout the body (hemorrhagic septicemia of Hueppe). The bacilli are short, non-motile, non-spore-bearing organisms. They exhibit bipolar staining and are Gram-negative. They do not liquefy gelatin. They are found in fowl cholera, swine plague, a similar disease in cattle and in laboratory test animals. The bacillus of bubonic plague seems to be closely related to the bacteria of this group.

BACILLUS OF CHICKEN CHOLERA.

(*Bacillus Avisepticus* or *Pasteurella Avicida.*)

In 1880, Pasteur carried on some fundamental studies on the bacillus of chicken cholera. He made the first bacterial vaccine with this bacillus. The bacillus was isolated from a widely disseminated acute disease of fowls and smaller birds.

Characteristics.—It is a short (0.5–1μ long) non-motile bacillus with marked polar staining. In general, its characteristics are similar to those of other members of this group.

Pathogenicity.—Pure cultures are very pathogenic for chickens and rabbits, less so for sheep, pigs and horses. Chickens are infected even by feeding minute amounts. A septicemia is produced which is rapidly fatal.

BACILLUS OF SWINE PLAGUE.

(*Bacillus Suisepticus* or *Pasteurella Suiseptica.*)

This organism is morphologically and culturally similar to the B. avisepticus. It differs in pathogenesis in that it produces naturally a disease of swine, characterized by a more or less chronic bronchopneumonia followed by septicemia. The gastro-intestinal tract is not markedly affected. The disease is generally fatal in young pigs. Thomsen[1] has reported 3 cases of infection in man.

BACILLUS OF BUBONIC PLAGUE (PASTEURELLA PESTIS).

Reference to plague is found in the Bible. Samuel describes the offering by the Philistines of golden images of the mice that marred the land to stay the pestilence. The fourteenth century saw the whole of Europe

[1] Hospitalstidende, 1918, **61**, 203.

stricken with this "black death." In India between 1896 and 1917 it is reported that the appalling number of 10,000,000 persons died of plague. Plague is endemic in some sections of the United States, Cali-

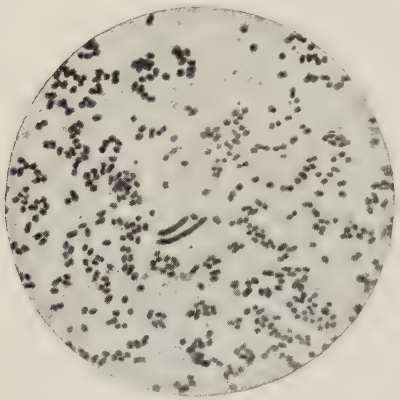


FIG. 138.—Bacillus pestis from agar culture. × 1100 diameters.

FIG. 139.—Bacillus pestis from bouillon culture. × 1100 diameters.

fornia, New Orleans and Texas. Among the most fatal forms of infection is that of the lungs. Pneumonic cases are not only very serious, but they readily spread the infection. The bacillus exciting the disease was discovered simultaneously by Kitasato and Yersin (1894) during an epidemic of the bubonic plague in China. It is found in large numbers in the seropurulent fluid from the recent buboes characteristic of this disease. In pneumonic cases the lungs and sputum contain immense numbers. It occurs in the blood in acute hemorrhagic cases and in fatal bubonic cases and therefore in the internal organs. It also occurs in malignant cases in the feces of men and animals. The relationship of rats to the disease was recognized in 1903, and the rat flea was established as the carrier in 1908.

FIG. 140.—Involution forms on salt agar. (Kolle and Wassermann.)

Morphology.—The bacilli in smears from acute abscesses or infected tissues are, as a rule, short, thick rods with rounded ends. The central portion of the bacillus is slightly convex. The bacilli are mostly single or in pairs. Bacilli in short chains occur at times. The length of the bacilli varies, but on the average is about 1.6μ (1.5μ to 1.7μ), breadth

0.5μ to 0.7μ. Besides the usual oval form, the plague bacillus has many exceptional variations which are characteristic of it. These pleomorphic forms are especially marked on 3 per cent. salt agar. In smears, especially from old buboes, one looks for long bacilli with clubbed ends (similar to involution forms, Fig. 140), yeast-like forms, and bladder shapes. Some of these stain faintly.

Staining.—They *stain* readily with the ordinary anilin dyes, and especially well with methylene blue, the ends being usually more deeply colored than the central portion; they do not stain by Gram's method.

Biology.—An aërobic, non-motile bacillus. Grows best at 30° to 35° C. Does not form spores. Grows on the usual culture media of slightly alkaline reaction. Does not liquefy gelatin. Grows well on *blood-serum* media. It grows rapidly on *glycerin agar*, forming a grayish-white surface growth. The bacilli appear, as a rule, as short, plump, oval bacilli, but a few present elongated thread forms which are very characteristic. In *bouillon* which is kept undisturbed a characteristic appearance is produced, the culture medium remaining clear while a pellicle forms on the surface from which projections sprout downward (stalactite formation) toward a granular or grumous deposit which forms on the walls and on the bottom of the tube. In bouillon and most fluid media the growth is in the form of short or medium chains of very short, oval bacilli, which look almost like streptococci. It does not coagulate milk, but produces a slight acidity. It produces no indol in peptone media.

Pathogenicity.—Plague is a rodent disease transmissible to man. The most important rodent is the rat. Mice may become infected. Other rodents may be infected and be the means of maintaining the disease in endemic areas, as the marmot in Thibet or the ground squirrel in California. The tarbagan was the primary source of infection in the epidemic of pneumonic plague in Manchuria (1910–11). Other species of rodents may later be found to be reservoirs of the disease.

These animals as well as guinea-pigs are easily infected artificially by feeding or application of the bacilli to the mucous membranes or to the skin. In the last, infection is sure to follow if a slight puncture or scratch is present. Monkeys and rabbits can also be infected. The bacilli may lose their virulence after prolonged artificial cultivation, but a strain Wilson has kept in our laboratory for many years still seems to be fully virulent for mice.

In *rodents* the disease may be either acute or chronic. A septicemia develops as a terminal event and fleas feeding at this time become infected. In *man*, the disease is bubonic, pneumonic or septicemic in type. Septicemia is the usual mode of termination of the bubonic and pneumonic types of the disease.

The diagnosis of natural infection in rats is made macroscopically, although occasionally the disease does occur with little and possibly no evident lesions. Rats may show only marked emaciation and anemia. In acute rodent plague the engorgement of the subcutaneous

bloodvessels and the diffuse pink color of the subcutaneous tissues and muscles is strongly diagnostic. In subacute plague the superficial lymph nodes are very much enlarged and frequently surrounded by edema or hemorrhagic areas. The spleen is very much enlarged and soft. The liver is mottled with small hemorrhages and yellowish punctate areas of necrosis. Generally, there is an excess of fluid in the pleural cavity. In the more chronic disease, abscesses of the peripheral lymph nodes or more commonly mesenteric or splenic purulent or caseous foci are found. Frequently the rats submitted for examination are badly decomposed but pure cultures of the plague bacillus may be readily obtained by applying the material from the lesions to the freshly shaven abdomen of a guinea-pig. The plague bacilli penetrate the skin through the slight scarification due to dry shaving, whereas the other bacteria do not, and a general infection results. At least the organs of all suspicious rats should be inoculated into guinea-pigs to verify the presence of plague bacilli.

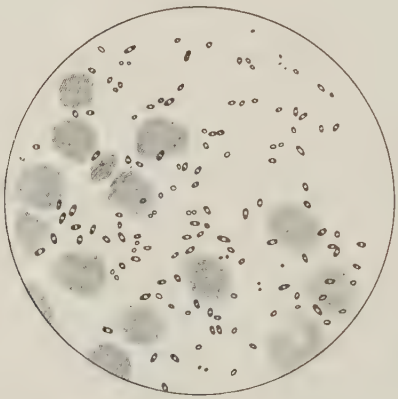


Fig. 141.—Bacilli in smear from acutely inflamed gland.

The guinea-pig usually shows symptoms of illness, about thirty-six hours after inoculation, and dies about the fourth or fifth day. At the site of inoculation there is usually a hemorrhagic exudate and the regional lymph nodes are swollen and surrounded by hemorrhagic infiltration. The spleen and liver are enlarged and congested. A pneumonia may be found. Large numbers of plague bacilli are present in the organs. All inoculated guinea-pigs should be killed and examined especially if inoculated with putrified material as putrefaction may lessen the virulence of the plague bacillus so that the disease in guinea-pigs may fail to kill.

Epidemiology.—The disease is maintained in the rodents. Although direct contagion may occur, the most important mode of transfer is by rodent fleas. The bacilli taken up with infected blood multiply in the digestive tract of the flea but the mouth parts seem not to remain infected. Fleas may remain infected for weeks or even one or two months. The mechanism of infection is probably the deposition of infected feces dur-

ing the act of feeding or regurgitation of infected blood from a previous meal. Plague bacilli can invade the tissues through a flea bite, thus if a non-infected flea is allowed to bite a rat and a drop of plague culture be placed on the bite infection results.

Bubonic plague in man is due to transfer of infection by means of fleas. The British Plague Commission proved conclusively that this is a fact. They allowed plague-infected rats to remain in contact with healthy rats in the absence of fleas and found that the healthy rat remained well, whereas, when fleas were placed in the cages the healthy rats became infected. They varied their experiments and corroborated their results. The pneumonic type may develop after infection by fleas due to blood invasion and pulmonary localization. Should this occur an outbreak of pneumonic plague may develop due to man-to-man contagion. This is most likely to occur during cold weather and under conditions of close contact. This form of disease is extremely contagious, with a mortality of 90 per cent. or more. The mortality of bubonic plague varies from 30 to 90 per cent.

As stated above, the fleas of rodents are the means of transfer of plague. All the fleas which infest rodents are not equally important in this regard. Flea surveys have shown that the incidence of plague and the incidence of Xenopsylla cheopis of rats runs parallel. This rat flea and Ceratophyllus fasciatus will bite man and given the right conditions the latter might be as efficient a means of plague transfer to man as the Xenopsylla cheopis. Temperature and humidity are the determining conditions as regards the incidence of rat fleas and it is only where these conditions are satisfactory that plague becomes permanently endemic.

Surveys carried out in the United States under the Public Health Service have shown that in the southern ports where plague existed Xenopsylla cheopis is the predominating rat flea during every month of the year. In northern ports where plague has never existed Ceratophyllus fasciatus is predominant and the Xenopsylla cheopis appear during only a few months of the year. Likewise the flea incidence is consistently high in the southern ports as compared with the northern ones.[1]

Other biting insects than fleas may possibly be a factor in infection, for example bed-bugs. It is possible that human vermin were a factor in the widespread outbreaks of "black death" during the middle ages.

Immunity.—Like typhoid infection, a single attack of the plague bacillus protects, with rare exception, from a second infection. Yersin, Calmette, and Borrel have succeeded in immunizing animals against the bacillus of bubonic plague by the intravenous or intraperitoneal injection of dead cultures, or by repeated subcutaneous inoculation. They also succeeded in immunizing rabbits and horses, so that the serum afforded protection to small animals, after subcutaneous injection of virulent cultures, and even cured those which had been inoculated,

[1] Public Health Reports, 1925, **40**, 1909.

if administered within twelve hours after injection. It also contains specific agglutinins which may be made use of in identification of suspected cultures.

Plague Bacillus Vaccine.—A relative immunity against plague is apparently established by vaccination, but is of short duration, not much over six months. Vaccination has been done on a large scale in India by Haffkine who used specially grown broth cultures. Others use saline suspensions of agar grown organisms. Reactions may be marked.

Serum Treatment.—Yersin obtained an antiserum by injecting horses with increasing dose of B. pestis. Such an antiserum has some antitoxic action, but is mainly antibacterial in action. Its therapeutic effect is not marked but is greatest when used very early in the disease.

Duration of Life Outside of the Body.—In cultures protected from the air and light the plague bacilli may live ten years or more (Wilson). In the bodies of dead rats they may live for two months. In sputum from pneumonic cases the bacilli lived ten days. Upon sugar sacks, food, etc., they may live six to fifteen days.

Resistance to Deleterious Influences.—The bacilli resemble the colon bacilli in their resistance to heat and disinfectants.

Bacteriological Diagnosis.—Material is obtained in bubonic plague by puncture or incision of a lymph node; in pneumonic plague the sputum is employed. Direct smears if plague-like bacilli are present are valuable in making a rapid presumptive diagnosis. Cultures and cutaneous inoculation of guinea-pigs should also be made. Blood cultures are frequently positive, especially late in the disease. In post-mortem examinations the heart blood and spleen should be examined. Wherever the material is badly contaminated or even decomposed the cutaneous method of inoculation of guinea-pigs should be resorted to.

Precipitin Reaction with Tissue Extracts.—Warner,[1] following the technic of Ascoli as applied to anthrax lesions, has found this method of considerable value. The method is of great advantage in making a rapid presumptive diagnosis and is also of value in old or decomposed lesions which are not adapted for bacteriological examination. Here again it must not be forgotten that the reaction is only quantitatively specific, and must be adequately controlled. A negative reaction is not conclusive.

Pasteurella Tularensis.—McCoy (1911) and McCoy and Chapin (1912) found an organism in a plague-like disease of California ground squirrels. They called that microbe Bacillus tularense after the name of the county (Tulare) where it was first found. Under the latest classification it is known as Pasteurella tularensis. It is a small, Gram-negative, non-motile, non-spore-bearing, pleomorphic, aërobic bacillus, with an optimum temperature of 37° C. an optimum pH range of 6.8 to 7.3. It ferments glucose, levulose, mannose and glycerol, forming no gas. It grows well on coagulated egg yolk and blood-glucose-cystin agar, but not on ordinary laboratory media. Bacillary, coccoidal and bipolar forms are noted.

[1] Jour. Hygiene, 1914, **14**, 360.

Since its discovery the intensive study of its characteristics and incidence made chiefly and exhaustively by Francis[1] has brought to light many interesting facts.

The bacillus is much more widespread than was thought. All rodents are susceptible but in the wilds it is found chiefly in rabbits and hares. The disease it causes is often called the rabbit disease. Francis named it tularemia. It is transmitted from rodents to man by the bite of an infected blood-sucking, insect fly (chiefly the Chrysopdiscalis) or tick (Dermacentor andersoni) or by the contamination of his hands or conjunctival sac with infected rabbits or other animals. Flies transmit the infection mechanically while ticks may transmit it through their eggs to the next generation, therefore they may be considered permanent reservoirs of the infection.

According to Francis, tularemia has been erroneously diagnosed from time to time by clinicians as influenza, septic infection, typhoid pneumonia, sporotrichosis, tuberculosis or undulant fever.

Francis says the following tetrad is diagnostic: (1) a history of having dressed or dissected a wild rabbit, or of being tick-bitten or fly-bitten; (2) a primary lesion of the skin in the form of a papule, followed by a persistent ulcer or a primary conjunctivitis, followed often by ulcers of the conjunctiva; (3) persistent glandular enlargements in the region draining the primary lesions, and (4) fever of from two to three weeks' duration.

The diagnosis may be proved (1) by obtaining an agglutination of Bacterium tularense by blood serum collected in the second week of illness and by noting an increase in the agglutination titer in serum collected a few days later or in the third week, or (2) by isolation of Bacterium tularense from guinea-pigs inoculated with material taken as early as the first week from the primary lesion or from the enlarged glands or the blood of the patient. Microscopic examination of cover-glass preparations taken direct from the patient is useless.

A gradual diminution in the amount of agglutinins takes place until at the end of the first year. The average titer of 21 cases was 1 to 140. Specific agglutinins remain for years in the blood of recovered patients and do not entirely disappear from any case, even ten, eleven, twelve, fourteen, fifteen, eighteen or nineteen years after recovery.

Francis says he does not know of any other disease in which an agglutination test will set the diagnosis right in such certain terms after so many years. By the employment of agglutination in conjunction with clinical evidence in 51 cases, Simpson reported in June, 1928, a continuous record of the unrecognized existence of tularemia in Dayton, Ohio, throughout the preceding twenty years.

Human tularemia serums may agglutinate Brucella abortus and Brucella melitensis, the causative agents of malta and undulant fever. "Of 570 human tularemia serums so tested, 129 showed such cross-agglutination, while 441 failed, even in a dilution of 1 to 10. Many

[1] The latest reports by Francis are Tularemia, The Atlantic Med. Jour., 1927, **30**, 337; and Jour. Am. Med. Assn., 1928, **91**, 1155.

of the latter group were of maximum anti-tularense titer (1280 to 2560). As a rule, a tularemia serum agglutinated Bacterium tularense in much higher dilution than it agglutinated Brucella abortus or Brucella melitensis, and the cross-agglutination of the latter organisms was much slower in developing in the water-bath. Exceptions to that rule were noted in 12 serums which, after standing in the ice-box over night, were found to have agglutinated Bacterium tularense, Brucella abortus and Brucella melitensis to the same, or to nearly the same, degree."

"Human undulant fever serums may show cross-agglutination of Bacterium tularense. Of 93 undulant fever serums so tested, 31 showed some degree of such cross-agglutination while 62 showed none. Among the latter group were serums which in dilution of 1280 to 2560 gave agglutination for undulant fever."

Francis reports four clinical types of tularemia from a study of 679 case reports:

1. *Ulcero-glandular Type.*—The primary lesion is a papule of the skin, later an ulcer, and is accompanied by enlargement of the regional lymph glands.

2. *Oculo-glandular Type.*—The primary lesion is a conjunctivitis and is accompanied by enlargement of the regional lymph glands.

3. *Glandular Type.*—There is no primary lesion at the site of infection, but there is enlargement of the regional lymph glands.

4. *Typhoid Type.*—There is no primary lesion nor is there glandular enlargement. (The word "typhoid" is used here in the sense of an absence of manifest external lesions and not as signifying the so-called typhoid state of mind.)

Reports of the widespread incidence of this bacillus are continuing to be published.

Among the animals added to the susceptible list are the ruffed grouse[1] and the ring-necked pheasant.[2]

[1] Green and Wade: Soc. Exp. Biol. and Med., 1928, **25**, 515.
[2] Green, Wade and Kelly: Soc. Exp. Biol. and Med., 1928, **26**, 260.

CHAPTER XXVIII.

THE GROUP OF HEMOGLOBINOPHILIC BACILLI AND BORDET-GENGOU BACILLUS.

(Genus Hemophylus).

THE INFLUENZA BACILLUS (HEMOPHYLUS INFLUENZÆ).

All bacilli that will not grow in pure cultures without the presence of hemoglobin or a similar substance are called hemoglobinophilic bacilli. All bacilli having this characteristic, that have been isolated and studied, resemble each other in several other particulars. That is, they are minute, faintly staining Gram-negative rods with a tendency to bipolar staining and the formation of delicate threads. They have not been found outside of animal bodies. They have been demonstrated in several diseases both of humans and of some of the lower animals. Claims have been advanced particularly that members of the group are the cause respectively of the epidemic disease called influenza, of the disease called acute contagious conjunctivitis or "pink eye," of the conditions called trachoma. Since the recent great pandemic of influenza the studies made of this group of organisms have altered our ideas of their relation to epidemic influenza.

Epidemics occurring periodically and possessing the same general characteristics as those classed under the name influenza can be traced back to the fifteenth century and probably existed at a much earlier date. Between epidemics endemic cases occur. We are not even certain that these epidemics are all started by the same virus. The last pandemic was probably the most widespread and severe of all.[1] The World War probably prepared the way for several foci at about the same time. The epidemic before this one seemed to start in eastern Russia in the fall of 1889. Between epidemics we have a varying number of cases diagnosed "influenza" especially numerous during the winter months. Many acute inflammations of the respiratory mucous membranes due to pneumococci and streptococci give symptoms similar to those supposed to be due to the influenza bacillus.

After numerous unsuccessful attempts, during the epidemic of 1889 and succeeding years, to discover the specific cause of influenza, Pfeiffer (1892) succeeded in isolating and growing upon *blood-agar* a bacillus which he found in the purulent bronchial secretion of patients suffering

[1] Soper: Science, 1918, n. s., **48**, 451; also Winslow and Roger: Jour. Inf. Dis., 1920, **26**, 185; Vaughan: Am. Jour. Hyg., 1921, Monograph No. 1.

from affections diagnosed as epidemic influenza, which he claimed was the cause of the disease. Canon, working at the same time, found a similar bacillus in the blood of several cases of the disease. Pfeiffer proved that in pure cultures this bacillus required hemoglobin for its growth. Though B. influenzæ was shown to have definite pathogenic powers, its specificity in epidemic influenza was not proved.

Pfeiffer's reasons for claiming that his bacillus, which he called Bacillus influenzæ (*Hemophylus influenzæ*), is the cause of epidemic influenza are as follows: (1) He found it in large numbers in every case he examined, sometimes apparently in pure cultures. (2) He found no other single variety of organism in every case. (3) He found it at the site of the lesion; that is, in the bronchial secretions in the living and, as well, in the deeper portions of the lungs after death. (4) He said that it disappeared from the secretions after the patient became entirely well. (5) He did not find it in other conditions. (6) He said that he had produced the disease irregularly in monkeys, and atypically in rabbits, and that he recovered his organism from the site of the lesion. He thus claimed to have practically fulfilled the postulates of Koch, which were supposed, at that time, to embody the proof of the specific pathogenic ty of an organism.

Fig. 142.—Influenza bacilli. × 1100 diameters.

We know now, however, that these "postulates" are not necessarily sufficient to prove that a given organism is specifically pathogenic; neither are all of them always essential for proof. We know that even the demonstration of specific antibodies, in the host, while constituting some evidence of an etiological relationship is not proof of it. And we know, contrary to Pfeiffer's first statement, that influenza-like bacilli, that cannot yet be differentiated from the Bacillus influenzæ by any known tests, are found in many conditions other than epidemic grip, sometimes definitely as secondary infections, *e. g.*, tuberculosis, measles, pertussis and sometimes apparently as primary infections, *e. g.*, meningitis, pink eye. And since the animal experiments were not clear-cut, and no group antibodies were demonstrated, and since other observers did not find influenza bacilli in all cases diagnosed as epidemic grip, doubts accumulated in regard to their specific action in this condition. (See below.)

Morphology of the Influenza Bacillus.—Very small, moderately thick bacilli (0.2μ to 0.3μ in thickness to 0.5μ to 2μ in length), usually occurring singly or united in pairs, and occasionally showing threads, are

found in spreads from the sputum and young cultures. In later cultures threads may be produced in great abundance. Some strains produce threads more rapidly and abundantly than others, and some show more globoid forms. No capsule has been demonstrated.

Staining.—The bacillus *stains* rather faintly with the ordinary anilin colors—best with dilute Ziehl's solution (water 9 parts to Ziehl's solution 1 part), or Löffler's methylene-blue solution, with heat. Giemsa's method stains them well and brings out the polar granules which sometimes develop in these bacilli. They are not stained positively by Gram's method.

Biology.—An aërobic, facultative anaërobic non-motile bacillus, does not form spores; no growth occurs with most cultures below 22° C., or above 41° C. Some strains ferment the monosaccharids and a few of these produce a slight amount of gas in glucose media (Wadsworth and Wheeler[1]). Some strains produce indol (Jordan,[2] Rivers[3]).

Cultivation.—This bacillus is best cultivated at 37° C. on special culture media containing hemoglobin (see Media). At the end of eighteen to twenty-four hours in the incubator at 36° C. very small colonies are developed, (1) on blood-pour agar plates, to the naked eye, as minute dew-drop colonies, and (2) on blood-drop agar plates, under the microscope (mag. 100 diam.), as small whitish areas embedded among the red-blood cells. The pneumococcus and streptococcus show a greenish coloration and rim. The influenza colonies are most typical on this plate. They often show characteristic "heapings" in the center, especially after forty-eight hours. When surrounded by blood cells, the margins of the colonies often appear irregularly scalloped, but on the edges of the blood mass the free margins of the colonies are usually entire, with an occasional indentation. It is difficult, however, to focus on a definite marginal line, as the margin seems to vanish suddenly on focussing. On blood-drop-vitamin agar plates high dilutions often show very large colonies of influenza bacilli, especially after forty-eight hours. Oleate-blood-agar (see Media) shows very characteristic colonies in forty-eight hours, and as this medium inhibits the growth of pneumococci and most streptococci, it is a very helpful medium to use in isolating the bacillus (Fig. 143). Fishings are made and planted on glycerin-veal-blood agar (chocolate medium, see Media). Cultures, if kept moist, may remain alive for a variable time. Transplants should be made at least once a week. Pfeiffer claimed that not only were the red blood cells the necessary part of the blood needed for the growth of the influenza bacillus but that it was the hemoglobin in the cells that was the essential element.

Though in pure cultures this organism will not grow without hemoglobin, or a similar substance, in the presence of certain other organisms it will grow abundantly for successive culture generations without blood. Neisser and others found that it would grow well with members of the diphtheria group, and with the pyogenic cocci. We have found (Williams

[1] Personal communication.
[2] Jour. Am. Med. Assn., 1919, **72**, 1542
[3] Bull. Johns Hopkins Hosp., 1920, **31**, 50

and Mishulow;[1] Williams and Povitzky[2]) that while it grows very abundantly in *mass* cultures with a number of other microörganisms, with staphylococci grown in *mass* cultures it dies out after a few successive transplants.

Much work has been done on the growth-stimulating substances necessary for the influenza bacillus Some of it is corroborative of earlier work (Davis,[3] Rivers[4] and others) some of it is newer (Avery and co-workers[5]). The influenza bacillus needs two substances for its growth, one, called the X factor by Avery, is associated with the blood pigment, gives the peroxidase reaction and is not destroyed by moist heat at 120° C.; the second, called the V factor is less resistant to heat, resisting 100° C. for few minutes, but destroyed at higher tem-

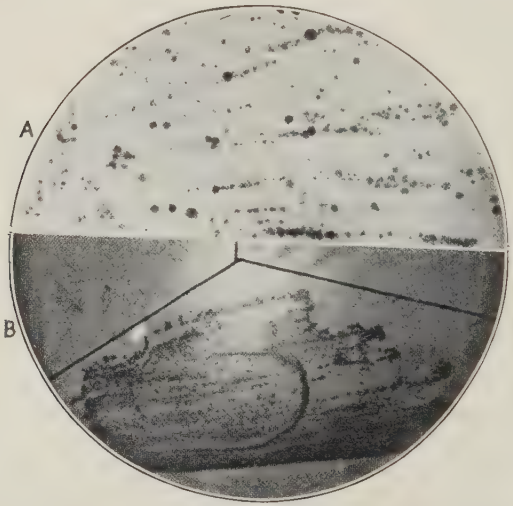


FIG. 143.—Bacillus influenzæ from throats grown on oleate-hemoglobin agar. *A*, throat culture from a normal person showing large colonies of B. influenzæ; thirty-six hours growth at 37° C. (only half plate shown). *B*, corresponding sectors are planted with the same organisms as in *A*, and show enhanced growth of B. influenzæ in the lower sector and complete inhibition of growth of pneumococcus and Streptococcus hemolyticus (only half plate shown). (Pritchett and Stillman.)

perature. It is either a co-enzyme or vitamin-like substance, while the X substance is like a biocatalyst. Certain plant tissues also seem to contain these two substances.

Resistance and Length of Life.—The influenza bacillus is very sensitive to desiccation; a pure culture diluted with water and dried is destroyed with certainty in twenty-four hours; in dried sputum the vitality, according to the completeness of drying, is retained from twelve to

[1] Jour. Inf. Dis., 1914, **14**, 261.
[2] Jour. Med. Res., 1921, **42**, 405.
[3] Jour. Inf. Dis., 1921, **29**, 171, 178, 187.
[4] Bull. Johns Hopkins Hosp., 1920.
[5] Jour. Exp. Med., 1921, 1923 and 1924.

forty-eight hours. It does not grow, and soon dies in water. In blood-bouillon cultures at 20° C. it retains its vitality for several weeks. In moist sputum it is difficult to determine the duration of its life, since the other bacteria overgrow and make it impossible to find it. It probably can remain alive for at least two weeks. For effect of heat and chemicals see Table under Disinfection.

Effect on Animals.—The bacillus of influenza is definitely though moderately pathogenic for some animals, especially the rabbit, and several observers have reported that such pathogenic power (from one blood-agar slant to $\frac{1}{10}$ of a slant) may be decidedly increased by successive passage through the susceptible animal. We have not been able to corroborate this. Guinea-pigs and mice are also quite susceptible to some strains. Pure, abundant cultures are always obtained from the heart after intraperitoneal or intravenous inoculation. Wollstein was able to produce cerebrospinal meningitis in the monkey by subdural inoculation of a strain from meningitis. Blake and Cecil[1] reported influenza-like attacks in monkeys after inoculation with B. influenzæ. Toxic products cause toxic symptoms in animals only when inoculated in comparatively large quantities. (Parker,[2] Huntoon.[3])

Immunity.—Short immunity may be established after an attack. Jordan[4] has reported observations made during the great epidemic. In some cases animals seem to be more sensitive to a second attack. The difference in strains may account for this. Hudson[5] reports that he was able to immunize white mice against twice the fatal dose of the culture. That rabbits may be hyperimmunized is shown in the process of obtaining antibodies.

Antibody Production.—That the various strains of hemoglobinophilic bacilli should be all of one type from the standpoint of antibody reactions could scarcely be conceived, but owing to the apparent inability to get clear-cut results in animals the question remained until recently an open one. Wollstein claimed a relationship between the strains that she isolated from meningitis and pneumonia, but she was only able to get low dilution agglutinations and did not make any absorption tests, neither did any one else.

Agglutination.—When we tested a very large number of strains isolated during the recent pandemic for specific agglutinins by the absorption test (see Chapter IX) we were surprised to find that practically each strain stimulated the production of abundant agglutinins, but that the specific agglutinins were usually absorbed only by the serum strain, very exceptionally by another. That is, similar strains were very infrequently encountered. This evidence of multiple strains from actual cases seems to be absolutely against the influenza bacillus being the cause of the pandemic, since a microbal pandemic must be due to later generations of the original virulent strain and it is not probable that we could miss a

[1] Jour. Am. Med. Assn., 1920, **74**, 170; Jour. Exp. Med., 1920, **32**, 691.
[2] Parker: Jour. Am. Med. Assn., 1919, **72**, 476.
[3] Huntoon, and Hannum: Jour. Immunol., 1919, **4**, 167.
[4] Jour. Inf. Dis., 1920, **26**, 463.
[5] Ibid., 1922, **30**, 433.

pandemic strain in so many cases or that the bacilli would become changed rapidly in the patient in their ability to produce specific agglutinins. Our work has been corroborated by a number of investigators.

Strains from meningitis isolated by Neal in our laboratory were found by Povitzky[1] to fall into a predominating group (50 per cent.) and several heterogeneous strains by the absorption of agglutinins test.

Complement Fixation.—Specific antibodies may be obtained in most rabbits, which definitely fix complement. The greatest difficulty in demonstrating the phenomenon of complement-fixation with this group of organisms is the preparation of suitable antigens. We have found that with the use of only small quantities of heated blood in the medium and by employing the method of shaking the cultures and then incubating overnight to help the autolysis, very satisfactory antigens have been obtained. Definite groups among these bacilli have not been demonstrated by this reaction.

Pathogenicity for Man.—The invasion of the body by the influenza bacillus is more widespread than was earlier supposed. It is probably more frequently secondary than primary. Very frequently the influenzal process invades portions of the lung tissue. In severe cases a form of pneumonia is the result, which is lobular, hemorrhagic and purulent in character and accompanied by symptoms which may be somewhat characteristic of influenza, or, again, almost identical with bronchopneumonia due to the pneumococcus. The walls of the bronchioles and alveolar septa become densely infiltrated with leukocytes, and the spaces of the bronchial tubes and alveoli become filled. The influenza bacilli are found crowded in between the epithelial and pus cells and also penetrate the latter. There may be partial softening of the tissues or even abscess formation. Bacilli are found in fatal cases to have penetrated from the bronchial tubes not only into the peribronchitic tissue, but even to the surface of the pleura, and in a few cases they have been obtained in pure cultures in the pleuritic exudation.

Presence in Other Parts of the Body.—Influenza bacilli are found at times in the blood during the early days of an acute infection, while there is marked fever and sometimes in severe cases in young children a septicemia develops before death. In some outbreaks they are found much more than in others. The difference depends again probably on there being different varieties. They are found at times in otitis media accompanying influenza and have been found in cases of meningitis (Wollstein[2]), in many cases of acute and subacute conjunctivitis (Williams[3]), frequently in sinusitis acute and chronic, and in several cases of peritonitis, appendicitis, and cystitis.

Toxic Effects.—The general, cerebral, gastric, and other symptoms produced are usually due to the absorption of the toxic products and of the specific organism, these poisons being particularly active in their effects on the central nervous system.

[1] Jour. Immunol., 1921, **6**, 65.
[2] Jour. Exp. Med., 1911, **14**, 73.
[3] Jour. Inf. Dis., 1914, **14**, 261.

Presence of Influenza Bacilli in Chronic Influenza and in Tuberculosis.—Ordinarily influenza runs an acute or subacute course, and not infrequently it is accompanied by mixed infections with the pneumococcus and streptococcus. Pfeiffer was the first to draw attention to certain chronic conditions depending upon the influenza bacillus. Bacilli may be retained in the lung tissue for months at a time, remaining latent a while, and then becoming active again, with a resulting exacerbation of the disease. Consumptives frequently carry influenza bacilli for years, not only in winter but also in the summer, when no influenza is known to be present in the neighborhood.

Influenza Bacilli in Other Diseases and in Normal Persons.—Persons suffering from bronchitis and all other diseases affecting the mucous membranes, especially of the upper respiratory tract, may harbor very many of these hemoglobinophilic bacilli. (Lord, Davis, ourselves and others). They may be found quite frequently also in apparently healthy persons.

Bacteriological Diagnosis.—In acute respiratory infections the bacilli are found as a rule *most abundantly in the nasopharynx*, therefore cultures should always be taken from this area as well as from the sputum. Material from the nasopharynx is collected on a swab, which is introduced, protected by a West tube, and planted on media similar to that given below.

Examination of Sputum for Influenza Bacilli.—1. Sputum coughed from the deeper air passages and not from throat scraping should be used.

2. The sputum should be expectorated into a sterile bottle, which should then be placed immediately in cracked ice to transport to the laboratory.

3. Blood-drop-agar plates should be made by placing a drop of fresh rabbit or horse blood, obtained aseptically, on the side of a hardened vitamin-blood-agar plate (see Media). Blood-pour plates and oleate-blood-agar plates (see Media) should also be used.

4. One of the more solid masses of the sputum should be taken from the bottle with sterile forceps and placed on a plain agar plate. A small portion of this mass should be separated with a sterile platinum needle and drawn through the blood on the blood-agar plate out in different directions. The larger part of what is left of this small portion is then placed in a similar manner over a second blood agar plate, and from this to a third, sterilizing the needle between the transfers. Similar seedings should be made on the other blood-media plates. The plates should be placed in the incubator at about 36° C. for twenty-four to forty-eight hours.

Similar cultures should be made from the nasopharyngeal swabbings.

5. After the plates are planted two smears should be made from the sputum, one stained by Gram and the other by weak carbol-fuchsin.

6. After twenty-four to forty-eight hours the plates are examined under low power. The influenza colonies use up the hemoglobin, and in parts of the blood-agar plate where the blood is of right thickness such colonies show as almost clear white areas surrounded by the red blood. With a higher power (No. 6 or 7 objective), if such areas seem to be made up of fine indefinite granulations, they are practically sure to be influenza colonies. Other characteristics have already been given.

7. Fishings from the influenza-like colonies should be planted on chocolate-agar-tubes, and if, after twenty-four hours in the thermostat, the resulting growth should consist of influenza-like organisms, plantings should be made on plain agar. The first generation on plain agar may show slight growth because of the blood carried over from the original tube, but the second generation should show no growth if the organism is the influenza bacillus.

Other Bacilli Resembling the Influenza Bacillus.—There are a number of bacilli which differ slightly in morphology and growth in culture from the characteristics of the typical influenza bacillus. These were grouped under the name "*pseudo-influenza bacilli.*" For example, the influenza-like bacilli found first in whooping-cough by Jochmann and others, Müller's "trachoma bacillus," Koch-Weeks' bacilli, the bacilli found by Cohen in meningitis, and those reported occasionally in other parts of the body—all of them seem to be so closely related that they should be considered one species or, at the most, varieties of one species until more specific characteristics can be demonstrated.

Hemophylus Hemolyticus.—The strains of influenza-like bacilli producing hemolysin and discovered by several of us at the same time, were first reported by Pritchett and Stillman,[1] and named by Rivers. They are probably non-pathogenic.

Relation of the Clinical Symptoms to the Bacterial Excitant.—There is no doubt that several infections are also included under the clinical forms of influenza. During interpandemic times and during epidemics of acute respiratory infections, irregular types of lobar pneumonia, and cases of bronchitis frequently have symptoms so closely alike that the nature of the bacteria active in the case is very frequently different from that supposed by the clinician. Thus in four consecutive autopsies examined by the writers the influenza bacillus was found almost in pure culture in one case believed, from the symptoms, to be due to the pneumococcus, and entirely absent in two of the three believed to be due to it. Except for these examinations the clinician would be of the opinion that he had clearly diagnosed bacteriologically the cases, while in fact he had been wrong in three of the four.

The striking symptoms in acute respiratory diseases are frequently due more to the location of the lesions than to the special variety of organisms producing them. In epidemics of influenza there are, of course, many cases which, on account of their characteristic symptoms, can be fairly certainly attributed to one type of organism. This is especially true in such a pandemic as the one just ended, where so many cases came down at the same time or in rapid succession, with such similar symptoms. But we must remain far from certain of the entity of the epidemics called influenza until we have proof of a specific cause.

Studies on the Specific Microbal Cause of Epidemic Influenza.—Among the many reports on the bacteriology of the last great pandemic, four claims for a specific etiological agent stand out in importance above the others: (1) That for a special streptococcus; (2) the claim for the influenza bacillus; (3) the claim for a filtrable virus; (4) the claim for a definite minute bacterium.

Evidence in Regard to Streptococci being the Initiating Specific Cause.—Gram-positive green-producing cocci in twos and chains of varying lengths have been reported by numerous observers as the cause of "flu" from the time of the 1889 pandemic, but the only evidence given in favor of them up to the present pan-

[1] Jour. Exp. Med., 1919, **29**, 259.

demic has been certain cultural similarities and the presence of apparently similar forms in large numbers in sputum and lungs of the majority of cases. During the early days of this pandemic, reports came from Europe that Gram-positive cocci were found, but no specific evidence was given. Here in this country Mathers (reported by Tunnicliff, November, 1918) found in 87 per cent of 110 cases at Camp Meade a "culturally characteristic green-producing streptococcus." Tunnicliff tested the opsonic power of the serum of four influenza patients and ten pneumonia cases against three strains and found increased opsonins as the patients recovered in the four influenza cases and in two of the pneumonias. Four more patients were tested in Chicago, with positive results. Two control strains (Micrococcus catarrhalis and Streptococcus hemolyticus) were used. These results are suggestive, but too few strains, cases and controls were used.

Rosenow claims to have found a similar streptococcus, though he describes the cultural characteristics somewhat differently, emphasizing their variability. He states that several organisms have in this pandemic shown selective affinity for the lungs, and chief among them is this streptococcus. He bases his claim for specificity upon the production of similar lesions in guinea-pigs when inoculated into the trachea and on the production of agglutinins in a horse, but he has not controlled his agglutination tests sufficiently by absorption of agglutinins, and until that is done nothing can be said for specificity of type.

Evidence in Regard to the Influenza Bacillus being the Initiating Specific Cause.—Here, again, we must show identity of strain or we add no new evidence in favor of the influenza bacillus being the exciting cause of the pandemic. When we began our studies on the significance of B. influenza in this pandemic and found these bacilli in practically 100 per cent. of the cases, we immediately started investigations to show identity of strains.

As we have shown above, agglutinin tests controlled by absorption of agglutinins have given such clear-cut negative results that they have practically overthrown the evidence in favor of the influenza bacillus being the specific inciting cause.

The work of Cecil[1] and his co-workers simply added evidence to show that influenza bacilli may produce a pneumonia similar to that found in epidemic influenza.

Evidence in Regard to a Filtrable Virus being the Initiating Cause.—Here too we have to consider the question of differentiating between a common invader of the respiratory tract and a pandemic strain.

Foster several years ago claimed that common colds are due to a filtrable virus. Some observers early reported a very few experiments indicating the possibility of a filtrable virus being present in this pandemic. Rosenow and Keegan had negative results in very small series of human inoculations. Then McCoy and his co-workers carried on two series of experiments with negative results, though these were made too late in the pandemic to draw positive conclusions.

Others reported positive cultures ("globoid bodies") by "Noguchi's method" and positive results in monkeys and some other animals, but some of these claims have been withdrawn.

Work on a Special Bacterium.—Olitzky and Gates[2] reported a series of studies on the isolation of a minute, filter-passing anaërobic, called by them Bacterium (Dialister) pneumosintes, obtained from the sputum early in a few cases of clinically typical influenza and from the lungs of rabbits inoculated with the sputum. No other investigator has been able to find a similar organism presenting evidence of relationship to epidemic influenza.

These studies on the specific initiating cause of the 1918–1919 pandemic of influenza may be summed up as follows: (1) The influenza bacillus is not the initiating cause of the pandemic; (2) too little

[1] Jour. Exp. Med., 1920, **32**, 691, 719, and Jour. Inf. Dis., 1921, **28**, 201.
[2] Jour. Exp. Med., 1921, **33**, 125, 361, 713, **34**, 1.

minute work has been done on streptococci and on a filtrable virus to give evidence for or against a member of either group being the specific etiological factor in the pandemic. The work of Olitzky and Gates has not been corroborated.

Vaccine Treatment.—Prophylactic Vaccination.—During the great epidemic the degree of reported success was directly proportional to the degree of the reporter's failure to critically control and analyze his figures. Wherever an experiment was carried out under perfect conditions of control and analysis the results indicate complete failure not only to prevent the disease but to modify the incidence of complications and their severity.[1] As far as we can determine, the disease when introduced in any group of persons, was most severe and more frequently complicated by pneumonia in the earliest cases. The tendency then was for the disease to diminish in severity. In any group from one-sixth to one-fourth or more would become infected, the remainder apparently being immune, although sporadic cases or even a recrudescence of a considerable number of cases might develop among the latter after the main wave of the outbreak was over. The apparently brilliant results of vaccination were obtained when vaccination was started at or near the peak of the case wave. It did not seem to make much difference what vaccine was used under these circumstances. In one instance the personnel of a manufacturing firm reported to us the startling results following only one dose of a commercial mixed vaccine (so-called common cold prophylactic vaccine). An analysis of the figures showed clearly that the peak of incidence had been reached when the vaccine was given. Similar results have been observed by us in the vaccination of large military groups, giving several billions of influenza bacilli. Even where an unvaccinated control group was not retained for comparison, the relative frequency of disease after vaccination is added and strong evidence of the failure of the vaccine. Vaccines of B. influenzæ isolated in this epidemic, either alone or in combination with pneumococci, streptococci, and other varieties such as staphylococcus, micrococcus catarrhalis, etc., were employed. The dosage was high in most instances totalling 5 billion or more. As most of these varieties belong to *practically* heterogeneous groups,[2] which we now know to be the case with the influenza bacillus, there is little if any theoretical basis for the hope that such vaccines will prevent the disease or its complications in any considerable number of persons. At the present time one must remember that we have no adequate evidence that we know the organism which is the primary cause of influenza.

Therapeutic Use.—Vaccines,[3] usually of mixtures of the influenza bacillus and the various cocci, have been employed with apparent success especially in the bronchopneumonias. Some of the comparisons of the severity and mortality among vaccinated and unvaccinated are

[1] McCoy: Jour. Am. Med. Assn., 1918, **71**, 1997. Minaker and Irvine: Jour. Am. Med. Assn., 1919, **72**, 847. Rosenau: Jour. Am. Med. Assn., 1919, **72**, 31.

[2] The report of Rosenau does not change our opinion in this regard.

[3] Wynn: Lancet, 1918, **2**, 874. Roberts and Cary: Jour. Am. Med. Assn., 1919, **52**, 922.

unjustified, however, as the cases compared were not comparable in point of time. As with prophylactic vaccination the importance of comparing only simultaneous controls was lost sight of. As such beneficial results as were obtained came on promptly, also because the vaccine was probably not specifically related to the infecting types[1] and suitable controls were not used one is led to believe that if any benefits were obtained they were due to non-specific protein action.

The use of autogenous vaccines where evidence points strongly to the etiological importance of B. influenzæ has given at best only suggestive results.

Dose.—Initial dose 50,000,000, later doses to 5,000,000,000.

Antiserum.—This has been used in cases of cerebral spinal meningitis by Neal of our Laboratory with apparently good results.

INFLUENZA-LIKE BACILLI IN CONJUNCTIVITIS (INCLUDING TRACHOMA).

The Koch-Weeks' Bacillus.—A bacillus was first observed by R. Koch in 1883 while making certain investigations on inflammation of the eye occurring during an epidemic of cholera in Alexandria. A similar bacillus was later, in 1887, more specifically described by Weeks[2]

FIG. 144.—Koch-Weeks' bacillus from "pink-eye" — third generation. × 1000 diameters. (Weeks.)

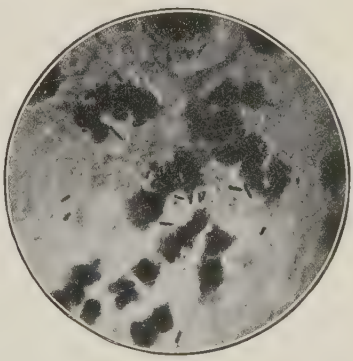


FIG. 145.—Secretion of mucopus from conjunctiva in "pink-eye." × 1000 diameters. (Weeks.)

in New York. Weeks obtained it in cultures growing with the xerosis bacillus from cases of "pink-eye," or acute contagious conjunctivitis. Morax stated that he was able to obtain pure cultures on hemoglobin-free media only until the third culture generation. Others state that on human serum or hydrocele fluid they have obtained growths for many generations. Kamen concludes that it is a strict hemoglobinophile.

[1] Snyder: New York Med. Jour., 1918, **108**, 843.
[2] New York Med. Rec., 1887, **26**, 571.

Our studies led us to agree with this last conclusion. The successive cultures obtained with some sera are probably due to the presence of hemoglobin in amounts too small to be easily detected, but large enough to allow growths of hemoglobinophilic bacilli. The few differential points claimed between this bacillus and influenza bacilli do not hold (Williams[1]) and so the question of their relationship is not settled. The general description of the influenza bacilli applies equally to both organisms. Absorption of agglutinins has not been tried sufficiently to draw conclusions as to there being special groups among those causing acute contagious conjunctivitis.

Other Microörganisms in Conjunctivitis.—Many organisms are found in diseases of the eye, but few of these present evidence of specific etiology.

Morax-Axenfeld Bacillus.—In certain subacute inflammations of the conjunctiva, especially noticeable about the angle of the eyes (angular conjunctivitis), Morax (1896) and later Axenfeld found a bacillus which they consider the cause of the disease.

Morphology.—Short (about 2μ long), thick, non-motile bacilli, generally in twos, but sometimes single or in short chains. They take the ordinary stains easily, but are decolorized by Gram's stain.

Cultures.—At 37° C. the bacilli produce a delicate growth on media containing blood or serum. Later cultures grow slightly on nutrient veal agar. They grow slightly, if at all, at room temperature.

Upon serum agar, they form delicate grayish colonies.

Upon Löffler's blood serum after twenty-four to thirty-six hours the growth appears as an indentation of the medium due to liquefaction. This liquefaction continues slowly for a variable time.

In ascitic broth cloudiness is produced within twenty-four hours.

Pathogenicity.—Lower animals so far have shown themselves refractory. In human beings inoculations of pure cultures have produced subacute conjunctivitis.

Bacillus of Nedden.—This bacillus has been found by Nedden in certain ulcers of the cornea and is supposed to be the etiological factor in this disease.

Morphology.—Small (usually less than 1μ long) rather slender, single, sometimes slightly curved, non-motile bacilli. They may occur as diplobacilli, but they do not form chains.

They stain easily, sometimes faintly at ends. They are decolorized by Gram.

Cultures.—They are easily grown on all laboratory media. Upon agar, within twenty-four hours they produce rounded, raised, translucent, slightly fluorescent colonies, which are more or less confluent and, under the low power, are rather coarsely granular. Upon *potato* they form a thick, yellowish growth. *Milk* is coagulated; *gelatin* is not liquefied; dextrose is fermented without gas. No indol is produced.

Pathogenicity.—Pure cultures inoculated in the cornea of guinea-pigs produced ulcers.

Trachoma.—Many studies have been made on the etiology of trachoma (progressive follicular inflammation of the conjunctiva followed by cicatrization) and allied conjunctival affections. Halberstädter and Prowazek (1907) state that the cause of trachoma is a small germ which grows in a characteristic way in the conjunctival epithelial cells. The organism itself, they say, is so small that at first it cannot be seen, only the mantle which it produces is demonstrable. This stains blue with Giemsa, and as the organisms grow in bunches, one sees at first in the neighborhood of the nucleus only a bunch of small, blue, coccus-like bodies. The organism finally appears as a minute red granule within the blue body. As it continues to increase in numbers and size, the blue

[1] New York Med. Jour., 1912, **95**, 540.

mantles finally disappear, leaving a mass of small rounded or slightly elongated red bodies. The bodies are only found in the early acute cases. Prowazek named them *Chlamydozoa* on account of their mantle, and thinks they should occupy a place between bacteria and protozoa.

Our studies[1] given below show that there is similarity between these inclusions and nests of growing hemoglobinophilic bacilli.

The Koch-Weeks' bacillus has been frequently reported as occurring in trachomatous eyes, from the time of Koch (Collins, Morax, Müller, etc.). Markus states that the Koch-Weeks' bacillus is the cause of "Schwellungskatarrh" (our papillary conjunctivitis). Müttermilch goes further in declaring that "often repeated infection with the same microörganism, *e. g.*, bacillus of Koch-Weeks, produces a series of exacerbations on an already inflamed conjunctiva and finally produces the picture of trachoma."

Müller, who isolated hemoglobinophilic bacilli from the largest series of trachoma cases reported, had positive results chiefly in his "acute trachoma" cases. Müller thought at first that his bacillus was the cause of trachoma, but others thought that it was an influenza bacillus and had nothing specifically to do with trachoma (Nedden, Morax and others).

We, however, agree partly with both workers. We have demonstrated the continued presence of hemoglobinophilic bacilli in cases showing successively acute, subacute and chronic inflammation of the conjunctiva and their increase in numbers during acute exacerbations of chronic cases. We have also shown that the bacilli found in cases of "pink-eye" are indistinguishable, thus far, from hemoglobinophilic bacilli found in the chronic cases. Furthermore, we have pointed out a morphological relationship between hemoglobinophilic bacilli and trachoma inclusions.

In studying closely the morphology in cultures of these bacilli, we were struck by the fact that they frequently grew in more or less dense clumps of extremely minute and irregular coccoid forms. This led us to the conclusion that possibly they form the Prowazek inclusions found in trachoma, and when we found that these bacilli and the inclusions were found coincidently and repeatedly in so many cases diagnosed as acute papillary trachoma (called by us papillary conjunctivitis), the possibility became a probability and we proceeded to study the morphology of the cultures more minutely.

The morphology varies somewhat with the age of the inoculated culture, the number of culture generations, the kind of medium and the strain. After forty-eight hours' incubation the forms become somewhat more irregular. Then in tbree days most of the bacilli have become extremely minute, many showing only as reddish granules (the "elementary bodies of Prowazek"), while scattered through the culture are swollen spheroidal bodies taking a fainter clear blue stain (the larger "initial bodies of Prowazek"), in some of which are minute reddish granules (more of the "elementary bodies of Prowazek"). A number of irregular light blue bodies are also scattered through the culture. Where the bacteria are densely grouped more red granules may appear in the center of the group than at the periphery and more blue bodies at the periphery than in the center. In short, all of the changes described by Prowazek and others as characteristic of trachoma inclusions are seen in the growing cultures of these hemoglobinophilic bacilli.

Similar day-to-day studies were undertaken with the other types of bacteria found most frequently in the eyes diagnosed as trachoma, *e. g.*, streptococci, staphylococci, gonococci, a minute Gram-negative, non-hemoglobinophilic bacillus not before described, found in a few cases of papillary conjunctivitis, xerosis bacillus and Micrococcus tetragenus, but in none of these varieties except the gonococcus were similar changes found in the same marked degree.

The fact that the gonococcus cultures showed such definite appearances similar to the trachoma inclusions led us to make a special study of a series of ophthalmia neonatorum cases.

[1] Jour. Inf. Dis., 1914, **14**, 261.

In all of the inclusion cases where gonococci are found, apparent transition forms between gonococcus and inclusion are very evident, and we find from a further microscopic study of these slides that the inclusions on the whole present certain characteristics different from those found in our series produced, according to our hypothesis, by nests of growing hemoglobinophilic bacilli.

From this comparative study of "inclusions" and cultures we have reached the following conclusion:

In many cases of "papillary conjunctivitis" and a certain number of cases of opthalmia neonatorum, as well as in a certain number af cases of inflammation of the mucous membranes of other parts of the body (*e. g.*, vagina, urethra), the trachoma inclusions found are due to one or more varieties of hemoglobinophilic bacilli; in a certain number of cases of gonorrheal ophthalmia as well as in gonorrheal inflammation of the mucous membranes of other parts of the body, the trachoma inclusions are due to the gonococcus. According to one of the later reports of Leber and Prowazek and the reports of Noguchi and Cohen,[1] and Noguchi,[2] certain inclusion conjunctivitis cases may be caused by microorganisms other than the two mentioned above.

THE BORDET-GENGOU BACILLUS (B. PERTUSSIS.)

In 1906 Bordet and Gengou[3] announced that they had discovered the etiological factor of whooping-cough to be a small bacillus found in predominating numbers in whooping-cough sputum. To this organism they gave the name Bacillus pertussis. Their claim that this bacillus is the real cause of whooping-cough they based upon their results with the complement-fixation test which they had been first to describe.

Morphology.—The pertussis bacillus is a short, oval rod, varying in size from about 0.2μ to 0.3μ in diameter and from 0.5μ to 2μ in length. It occurs singly, sometimes in twos joined at the ends, and very exceptionally in short chains.

Motility.—It is non-motile.

Staining.—The pertussis bacillus is decolorized by Gram's method. It is stained faintly by the ordinary anilin dyes. Bipolar staining is demonstrated very well by Gram's method and by toluidin blue (see Stains.)

Cultivation.—The pertussis bacillus grows best at 35° C. to 37° C. It grows slowly at room temperature.

It is aërobic, facultative anaërobic. When first isolated in pure culture it grows best upon the glycerin-potato-blood-agar media recommended by Bordet and Gengou. In later generations it grows more or less capriciously upon the ordinary culture media. Povitzky[4] has found that these bacilli grow well on a definitely acid Bordet-Gengou medium (pH 6.6). As this medium inhibits the growth of influenza bacilli, the isolation of the pertussis bacillus is made easier.

Unadjusted Bordet-Gengou between 6.1 to 5.8. For isolation the medium is adjusted by acetic acid to pH 5.0.

Unadjusted Bordet-Gengou is used for subsequent growth.

Isolation.—The sputum should be collected from the patient during the early stage of the disease, best in the first week. The thick grayish

[1] Arch. Ophthal. 1911, **40**, No. 1.
[2] Bull. New York Acad. Med., 1927, **3**, 395; Jour. Am. Med. Assn., 1927, **89**, 739.
[3] Ann. de l'Inst. Past., 1906, **20**, 573.
[4] Jour. Inf. Dis., 1923, **32**, 8.

portion of the sputum should be selected for the culture. This material is streaked over the surface of a plate of acid Bordet-Gengou medium. The plates are incubated at 35° C. to 37° C. In forty-eight hours to three days very minute, discrete, elevated colonies appear. When these colonies occur in abundance, the blood at their periphery is lighter red. In pure cultures this lightening of the blood is marked and may appear as hemolysis.

Identification.—(*a*) **By Culture.**—There are other bacilli occurring in whooping-cough sputum so closely resembling the pertussis bacillus in morphological and staining characteristics that they cannot be distinguished in smears. These bacilli, however, can be differentiated by their growth upon various culture media. The most important of these organisms is the influenza bacillus, which requires hemoglobin or two special substances for its growth (see above). There is frequently found a Gram-negative bacillus which makes a profuse growth upon all media from the first generation in pure culture. The following table gives the chief points of differentiation:

	Growth on Bordet-Gengou plates.	Bordet-Gengou slants.	Coagulated horse-blood slants.	Glycerin-ascitic-agar slants.	Plain agar slants.
B. pertussis . . .	Lightens the medium	First generation: in pure culture abundant non-spreading moist growth in twenty-four hours.	After several generations: abundant tenacious growth in forty-eight hours.	After several generations: abundant tenacious growth in forty-eight hours.	After several generations: tenacious growth occurs slowly.
B. influenzæ . . .	Darkens the medium. (No growth on acid Bordet-Gengou.	First generation: delicate growth	First generation: abundant moist spread in twenty-four hours	Never grows	Never grows.
Intermediate group of Gram-negative bacilli	Markedly lightens the medium	First generation: abundant moist spread in twenty-four hours	First generation: profuse moist spread in twenty-four hours	First generation: profuse moist spread in twenty-four hours	First generation: profuse moist spread in twenty-four hours.

(*b*) **By Agglutination.**—Agglutinins are easily obtained in rabbits for the B. pertussis, and by absorption tests we[1] showed (1916) that all strains tested then probably belong to the same type. Since that time Krumwiede and Mishulow[2] have found that there are at least two types, one of which occurs infrequently. It is not easy to diagnose a human case of whooping-cough by this method since agglutinins are not produced to any extent during the natural disease.

(*c*) **Complement Fixation.**—Bordet and Gengou regarded the complement fixation as the main support for their claim of the specificity of their organism. They report positive complement fixation in the majority of human cases tested by them. Other investigators, however, have reported negative tests of complement fixation.

[1] Povitzky: Arch. Int. Med. 1916, **17**, 279.
[2] Jour. Inf. Dis., 1923, **32**, 22.

The irregular reports given of the complement-fixation test in human beings may be accounted for by different methods used without making comparative studies and sufficient controls. The question of the best method of making the complement-fixation test is still undecided (Chapter XIV). Different observers have used different methods of preparing both antigen and serum and have employed different hemolytic systems. In experimental animals, specific antibodies are produced which give a positive complement-fixation test.

Pathogenicity.—The specific pathogenicity of the pertussis bacillus for man still lacks proof, though the evidence is strongly in favor of this organism being the cause of pertussis. Several investigators have reported its pathogenicity for monkeys, dogs, rabbits, and guinea-pigs.

Mallory[1] claims that a certain number of bacilli between the cilia of the epithelial cells in the trachea and bronchi constitute the specific lesion. He claims that he has fulfilled Koch's laws by the finding of these bacilli in experimental animals in the same situation and by the recovery of the culture from these animals. He acknowledges, however, that his results in animals are complicated by the fact that these animals are frequently infected by the Bacillus bronchisepticus (quoted as the cause of distemper in dogs) which is morphologically similar to the pertussis bacillus and that it apparently has the same power (as Theobald Smith and others have pointed out) to cling to the cilia of the epithelial cells in the respiratory tract. In human beings Mallory states, judging from the examination of hundreds of controls, only pertussis cases showed this lesion.

Vaccine Treatment.—Prophylaxis.—Some protection is conferred but it is difficult to judge of the degree with the data available. Hess vaccinated 244 children and 20 developed the disease, whereas of 80 equally exposed children 59 developed the disease. These results warrant its application, especially as the procedure is innocuous. We have made very extensive trials of the vaccine with a suggestion of slightly favorable results.

Dosage.—The initial dose should be 2 billions and the second and third 4 billions.

Therapeutic Use.—Hess could see no influence on the disease even where both prophylactic and therapeutic vaccines were given. It is difficult to correlate these results with the reports of others that the number of paroxysms and the duration of the disease is lessened. In some instances a prompt amelioration has been reported, so prompt as not to be explained on the basis of specific antibody production.

Dosage.—For children over one year 500 million, 1 billion and 2 billion at two-day intervals, is recommended. Children under one year receive half these doses. If, after several days, improvement is not marked, further injections may be given. Some advise doses up to 5 billion or more.

Recently we have prepared our vaccine by a method that allows of

[1] Mallory and Horner: Jour. Med. Res., 1912, **27**, 115; 1913, p. 391.

formation of a hypothetic toxin. The organism is grown on an unadjusted Bordet-Gengou medium for three days, washed off rapidly with a normal salt solution containing 0.5 per cent. phenol, and kept in the incubator at 36° C. for twenty-four hours, at which time the vaccine should be sterile. The undiluted vaccine usually contains about 50 billion bacilli to the cubic centimeter.

THE BACILLUS OF SOFT CHANCRE.

This bacillus was first specifically described and obtained in pure culture by Ducrey in 1889. An experimental inoculation is followed in one or two days by a small pustule. This soon ruptures and a small round depressed ulcer is left. About this other pustules and ulcers develop which tend to become confluent. The base of the ulcer is covered with a gray exudate and its edges are undermined. There is no induration such as in the syphilitic chancre. The secretion is seropurulent and very infectious. The process usually extends to the neighboring lymphatics, which become swollen, and may result in abscesses. These are known as "buboes."

Morphology.—About 1.5μ long and 0.4μ thick, growing in cultures often in chains and sometimes twisted together in dense masses.

It *stains* best with carbol-fuchsin, and shows polar staining. It is Gram-negative.

Cultural Characteristics.—The following method of cultivation has given good results: Two parts of liquefied agar at 50° C. are mixed with one part human, dog, or rabbit blood. The blood from the cut carotid of a rabbit may be allowed to run directly into the agar tube, to which the pus from the ulcerated bubo is then added in proper proportion, and the whole placed in the incubator at 35° C. The pus may be obtained by puncture and aspiration from the unbroken ulcer, or if the ulcer is already open it is first painted with tincture of iodine and covered with collodion or sterile gauze. After twenty-four to forty-eight hours, some pus having collected under the bandage, inoculations are made from it. The bacillus grows well also in uncoagulated rabbit-blood serum or in condensation water of blood agar. In twenty-four to forty-eight hours, on the surface of the media, well-developed, shining, grayish colonies, about 1 mm. in diameter may be observed. The colonies remain separate, but they increase in numbers after further transplantation. The best results are obtained when the pus is taken close to the walls of the abscess. Smears show isolated bacilli or short parallel chains with distinct polar staining. Teague and Deibert[1] have reported good results with clotted rabbit's blood heated at 55° C. for fifteen minutes. Cultures grown in it for twenty-four hours at 37° C. may then be kept alive in the refrigerator for from three to five weeks. All ordinary culture media so far tried have given negative results, and even with the media described, development is difficult and sometimes fails entirely.

[1] Jour. Med. Res., 1922, **43**, 61.

The characteristic soft chancre is produced in man after inoculation of cultures. Animals in general cannot be infected, but positive results have been obtained with monkeys and cats.

The organisms are especially characteristic in the water of condensation from blood agar, the bacilli being thinner and shorter, with rounded ends; sometimes long, wavy chains are found. In rabbit-blood serum at 37° C. a slight clouding of the medium is produced and small flakes are formed, consisting of short bacilli or moderately long, curved chains, showing polar staining.

The bacillus of soft chancre possesses but little resistance to deleterious outside influences. Hence the various antiseptic bandages, etc., used in treatment of the affection soon bring about recovery by preventing the spread by chancre.

CHAPTER XXIX.

GLANDERS BACILLUS (PFEIFFERELLA MALLEI).

The Bacillus mallei was discovered and proved to be the cause of glanders by several bacteriologists at almost the same time (1882). Bouchard, Capitan and Charin obtained it in mixed cultures, while it was first accurately studied in pure culture by Löffler and Schütz. It is present in recently formed nodules in animals affected with glanders, in the nasal discharge, in pus from the specific ulcers, etc., and in the blood in severe and fatal infections.

Morphology.—Small bacilli with rounded or pointed ends, from nutrient agar cultures, 0.25μ to 0.5μ broad and from 1.5μ to 5μ long; usually single, but sometimes united in pairs, or growing out to long filaments, especially in potato cultures. The bacilli frequently break up into short almost coccus-like elements (Fig. 146).

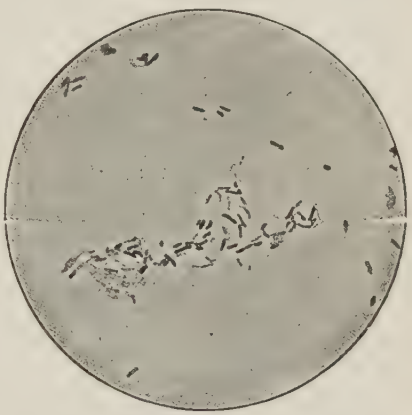


Fig. 146.— Glanders bacilli. Agar culture. × 1100 diameters.

Staining.—The bacillus mallei *stains* with difficulty with the anilin colors, best when the aqueous solutions of these dyes are made feebly alkaline; it is decolorized by Gram's method.

Biology.—A non-motile bacillus, whose molecular movements are so active that they have often been taken for motility. It is aërobic, but moderate multiplication occurs in the depths of culture media. Grows well on culture media at 37° C. Development takes place slowly at 22° C. and ceases at 43° C. The bacillus does not form spores. Exposure for ten minutes to a temperature of 55° C., or for five minutes to a 3 to 5 per cent. solution of carbolic acid, or for two minutes to a 1 to 5000 solution of mercuric chloride destroys its vitality. As a rule the bacilli do not grow after having been preserved in a desiccated condition for a week or two; in distilled water they may live twenty-five days. A solution of chlorinated lime, containing 1 part of free chlorine per 1000, is useful as a disinfectant of stables and utensils; it kills the bacillus in from one to two minutes. Strong sodium carbonate solution (washing soda) is also useful.

Cultivation.—It grows well at 37° C. on *potato-glycerin-veal agar*, an acidity of 1.5 to 2.5 (phenolphthalein), about pH 6.4, being the most favorable. Upon this medium, at the end of twenty-four to forty-eight hours, whitish, transparent colonies are developed, which in six or seven days may attain a diameter of 7 or 8 mm. On *blood serum* a moist, opaque, slimy layer develops, which is of a yellow-brown tinge. The growth on *potato* is especially characteristic. At the end of twenty-four to thirty-six hours at 37° C. a moist, yellow, transparent layer develops; this later becomes deeper in color, and finally takes on a reddish-brown color, while the potato about it acquires a greenish-yellow tint. In *bouillon* the bacillus causes diffuse clouding sometimes with a pellicle, ultimately with the formation of a more or less ropy, tenacious sediment. The broth should also be as acid in reaction as the potato-glycerin-veal agar above. The addition of potato juice to broth favors the growth of the organism. *Milk* is coagulated with the production of acid.

Pathogenicity.—The bacillus of glanders is pathogenic for a number of animals. Among those which are most susceptible are horses, asses, guinea-pigs, cats, dogs, ferrets, moles, and field mice; much less susceptible are sheep, goats, swine, rabbits, white mice, and house mice; cattle are immune. Man is susceptible and may develop either the acute or chronic form. Infection terminates fatally in about 60 per cent. of the cases. Doubtless many cases have not been recognized as glanders, but were mistaken for other diseases such as pyemia, rheumatism, typhoid and syphilis. (Fitch.[1])

When pure cultures of Bacillus mallei are injected into horses or other susceptible animals true glanders is produced. The disease is characterized in the horse by a rise in temperature and by the formation upon the nasal mucous membrane of ulcers which have irregular, thickened margins and secrete a thin, virulent mucus; the submaxillary lymphatic glands become enlarged and form tumors which are often lobulated. Other lymphatic glands become inflamed, and some of them suppurate and open externally, leaving deep, open ulcers; the lungs are also involved, and the breathing becomes rapid and irregular. *Acute generalized glanders* may cause death in one to six weeks. Young horses are especially susceptible. At *postmortem* the lungs show usually either tubercle-like nodules or pneumonic areas. Nodules may occur also in the liver, spleen and lymph glands. Of the bones, the ribs are the most often involved and contain caverns filled with a tenacious yellowish substance. In *farcy*, which is a more chronic form of the disease, circumscribed swellings, varying in size from a pea to a hazel-nut, appear on different parts of the body, especially where the skin is thinnest; these suppurate and leave angry-looking ulcers with ragged edges, from which there is an abundant purulent discharge. The lymphatics leading from these ulcers become inflamed, stand out as hot cords under the skin and from them new "farcy buds" may develop. Chronic cases may run on for years until an acute exacerbation due to overwork or adverse conditions brings death.

Isolation of Pure Cultures.—Attempts to isolate B. mallei by plating out the discharge from the nostrils or an open ulcer meet with little success since comparatively few bacilli are present usually. They are

[1] Cornell Veterinarian, July, 1914.

mixed besides with other bacteria which outgrow B. mallei on the plates. In such cases guinea-pig inoculations are useful if made as follows:

Straus Reaction.—A male guinea-pig is injected intraperitoneally with either emulsified material from a suspicious lesion or a suspension of a culture which has been obtained by plating. If B. mallei is present, tumefaction of the testicles occurs usually in two to five days with evidence of pus formation. This is the "Straus Reaction." The animal is then etherized and cultures made, under aseptic conditions, from the pus in the testicles. An objection to this method is that the animal may die of septicemia due to the injection of impure material, but if an uncontaminated specimen can be obtained, as from the lymphatic glands, this method is satisfactory according to most authors. Nevertheless, while a positive result is conclusive evidence of glanders, failure of the pig to develop lesions is not proof of the absence of B. mallei.

Of test animals guinea-pigs and field mice are the most susceptible. In guinea-pigs *subcutaneous* injections are followed in four or five days by swelling at the point of inoculation, and a tumor with caseous contents soon develops; then ulceration of the skin takes place and a chronic purulent ulcer is formed. The essential lesion is the granulomatous tumor, characterized by the presence of numerous lymphoid and epithelioid cells, among and in which are seen the glanders bacilli. The lymphatic glands become inflamed and general symptoms of infection are developed in from two to four weeks; the glands suppurate. Finally purulent inflammation of the joints occurs and death ensues from exhaustion. The formation of the specific ulcers upon the nasal mucous membrane, which characterizes the disease in the horse, is rarely seen when guinea-pigs are inoculated. In these the process of the disease is often prolonged or remains localized on the skin. Guinea-pigs succumb more rapidly to intraperitoneal injection, usually in from eight to ten days.

Attenuation of virulence occurs in cultures which have been kept for some time on artificial media and inoculation with such cultures may give a negative result, or, when considerable quantities are injected, may produce a fatal result at a later date than is usual when small amounts of a recently isolated culture are injected.

Mode of Spread.—Glanders occurs as a natural infection only in horses and asses. The disease is occasionally communicated to man by contact with affected animals, usually by inoculation on an abraded surface of the skin. The contagion may also be received on the mucous membrane. Infection has sometimes occurred as the result of accidents in bacteriological laboratories. It is transmissible also from man to man. Washerwomen have been infected from the clothes of a patient. The infective material exists in the secretions of the nose, in the pus of glanders nodules, and frequently in the blood; it may occasionally be found in the secretions of glands not yet affected, as in the urine, milk, and saliva, and also in the fetus of diseased animals (Bonome). Glanders may be present in apparently sound horses, sometimes taking a mild course and remaining latent for a considerable time. Therefore, horses

appearing healthy may spread the disease through the public drinking troughs and in blacksmith shops.

Immunity and Vaccination.—Attempts have been made to produce artificial immunity against glanders but so far with unsatisfactory results. Various workers, as Strauss, Fenger and also Ladowski, have reported the production of immunity in the smaller animals, such as dogs, cats and rabbits, by the injection of either living or killed cultures. In the horse, the most important animal from the economic standpoint, other observers have reported not only immunity but cures by the use of vaccine and also subcutaneous mallein; however, corroboration is still lacking. Such substances as mallein and vaccine when injected do produce immune bodies which can be demonstrated by the serodiagnostic tests. It is unfortunate, however, that the presence of these immune bodies in the blood does not indicate, necessarily, a practical immunity of the animal against infection. (Mohler and Eichhorn.[1]) The indiscriminate use of *vaccine* causes confusion in the blood tests of horses.

In man the therapeutic value of vaccine is not yet fully determined. But few cases so far have been reported—one by Bristow and White[2] and one by Cramp,[3] recovered after the use of an autogenous glanders vaccine. The use of *mallein* in man subcutaneously has been reported in six cases. (Robins[4]). In one it gave a reaction and in three it was of supposed benefit therapeutically.

Diagnosis of Glanders.—The chief methods (Mohler and Eichhorn[5]) are: physical examination; serodiagnostic tests—complement fixation and agglutination reaction; mallein reactions—eye, intrapalpebral and subcutaneous; "Straus Reaction" (inoculation of guinea-pigs with either suspected material or cultures); postmortem examination.

Physical Examination.—In horses those cases with clear-cut clinical symptoms offer little difficulty to the veterinarian. However, the easily applied eye mallein reaction should be used for confirmation. These two methods are sufficient under such circumstances.

Serodiagnostic Tests.—It is the latent or occult cases, showing only a little fever, or none, that require additional tests for diagnosis. Since these cases are frequently the distributors of the disease their early recognition and extermination is imperative. To detect these cases the application of the serodiagnostic reactions supplemented by the use of eye mallein is necessary.

Collection of Blood for Serodiagnostic Tests.—In obtaining blood from horses a large-sized hypodermic needle, which has been sterilized, is inserted into the jugular vein which has been brought into view by pressing the thumb upon it from below; the blood is allowed to flow through the needle into a sterile neutralized tube or flask, 8 to 10 c.c. being sufficient.

[1] Bull. U. S. Dept. Agriculture, 1914, No. 70.
[2] New York State Jour. Med., 1910, p. 236.
[3] Jour. Am. Med. Assn., 1911, **56**, 1379.
[4] Studies from the Royal Victoria Hospital, Montreal, 1906, **2**, 1.
[5] U. S. Dept. Agriculture, Bureau of Animal Industry, 1912, Circ. 191.

In the case of human beings, the median basilic vein at the bend of the elbow is used. Under aseptic conditions 5 to 10 c.c. of blood is drawn either by means of a sterile hypodermic syringe, or allowed to flow through a large hypodermic needle, as above.

Complement-fixation Test.—In 1909 Schütz and Schubert applied this method to the diagnosis of glanders. It gives excellent results, for it picks up 97 per cent. of positive cases according to Miessner and Trapp.[1] Its failures lie chiefly in the early stages of the disease, and for this reason it should be paralleled by the agglutination reaction. The combination of these two tests gives, according to Hutyra and Marck,[2] a percentage of 99 successful tests. The use of a mixed antigen for the detection of glanders is important.

Agglutination Reaction.—This reaction was first applied by MacFadyean (1896) who used the microscopic method as in the Widal reaction. Later Schütz and Miessner (1896) found the macroscopic method more practicable. In the early stages of glanders this method is most valuable. It picks up about 84 per cent. of positive cases (Anthony and Grund[3]), the failures occurring chiefly in old chronic cases.

Schütz and Miessner claim that a culture recently passed through a guinea-pig (once in three weeks) is essential for a good test fluid. In our hands cultures kept on properly made artificial media (potato-glycerin-veal-agar) do well if passed through a pig once in two months. Not every strain of B. mallei agglutinates well, consequently a suitable one must be chosen.

The *macroscopic agglutination* test may be carried out in several ways:

Incubator Method.—The procedure of Schütz and Miessner, with slight modifications, is as follows: A forty-eight-hour acid-glycerin-agar culture of B. mallei is washed off with normal saline solution containing 0.5 per cent. pure carbolic acid. This suspension is heated at 60° C. for two hours. It is then filtered through cotton, and enough of the carbolic salt solution added to reduce it to a faintly cloudy suspension. This should be standardized by comparing it with a known test fluid, if possible, and testing it with known negative and positive sera. This test fluid will keep in the ice-box for several weeks.

The serum (active) to be tested is then made up with normal salt solution to 1:40 dilution. From this the final dilutions of 1:500, 1:800, 1:1000, etc., are made by adding 0.24 c.c., 0.15 c.c., and 0.12 c.c. respectively to 3 c.c. of the standardized test fluid in each test-tube. The tubes are well shaken and incubated twenty-four to seventy-two hours with positive and negative control sera in the same dilutions. If a reaction occurs the upper part of the fluid is clear, while a veil-like sediment is found at the bottom. A strong positive reaction (1 to 1000) may occur in twenty-four hours. A negative reaction shows the sediment in a definite "button" at the bottom of the tube and the fluid above is cloudy.

With the *centrifuge method* of Miessner and others, cited by Mohler and Eichhorn,[4] the time factor is greatly reduced. The tests are incubated for a half-hour at 37° C., then centrifugalized at 1600 revolutions for ten minutes, and kept at room temperature for two hours before reading. This is done by looking down on the tubes from above toward a dark background. Indefinite reactions may be read the next day after standing at room temperature. Special

[1] Centralb. f. Bact., 1909, Band, **52.** [2] Ibid.
[3] Collected Studies, Bureau of Lab., City of New York, 1913, **7**, 291.
[4] U. S. Dept. Agriculture, Bureau of Animal Industry, 1912, Circ. 191.

tubes with perfectly rounded bottoms are essential for this method, and the dilutions are made up in only 2 c.c. of the test fluid.

The limit of agglutination (*regular methods*) in the normal horse is 1 to 500, most of the reactions occurring at 1 to 200 or 400. Since, however, some cases of chronic glanders do not react above 1 to 500, this reaction should be regarded with suspicion and checked by the complement-fixation test and the ophthalmic test; so also reactions below 1 to 1000. Reactions of 1 to 1000 are considered positive, some horses running up to 1 to 2000 or 3000. In practical work dilutions higher than 1 to 1000 are unnecessary.

Rapid Method.—Povitzky, of this laboratory, is applying the following method of macroscopic agglutination with time-saving results. Technic: A fresh culture grown for forty-eight hours on glycerin-potato-veal agar (2.5 acid to phenolphthalein) is washed off with a small amount of sterile normal salt solution. This thick milky suspension is heated for one hour at 70° C.; it may then be kept in the ice-box for two months or more. Whenever it is to be used, normal salt solution is added until the suspension is only faintly cloudy. This test fluid must be standardized by comparison with a known standard, etc., as described under the *incubator method* of agglutination. The active serum to be tested is then diluted with normal salt solution and added in suitable amounts to 3 c.c. of the test fluid (see procedure under incubator method). The dilutions, however, are carried higher—1 : 1200, 1 : 1600, 1 : 2000 usually. The various dilutions and controls are then placed in a water-bath of 37° to 40° C. for two hours. Very active reactions can be read as early as the end of the first hour; others at the end of the second hour. The tubes are then set in the ice-box overnight and any delayed reactions may be read next morning. Although this method is only a little more rapid in time than the centrifuge method, its easier technic and lack of complicated apparatus recommend it.

In reading the rapid method tests on horses a reaction is considered as positive which has double plus through 1 to 1600; as suspicious with double plus in 1 to 1200; as doubtful with double plus in 1 to 1000. Any reactions below 1 to 1000 are considered as negative.

These tests should always be checked by the ophthalmic-mallein test and the complement-fixation test.

In human cases a reaction by the "rapid method" (Povitzky) of 1 to 500 and above is considered positive. Normal human blood reacts seldom above 1 to 100, but it may reach 1 to 200 or even 400 in exceptional instances.

Mallein Reaction.—Mallein is like tuberculin in that it consists of glycerinated bouillon culture which contains the products of the growth of B. mallei. It was discovered by Kelnig, a Russian veterinarian, in 1890. (For the preparation of the two kinds of mallein—*eye* and *subcutaneous*, see below.)

The *ophthalmic-mallein test* is the Federal test for the interstate shipment of horses. The simplicity of the application of eye mallein, the short time –twenty-four hours—required, and the comparatively easy reading of results, after a suitable experience, make it possible for the veterinarian to apply prompt tests on suspected horses.

In healthy horses the error of this test has been shown (Schnurer[1]) to

[1] Proc. Tenth International Veterinary Congress, London, 1914.

be only 0.39 per cent., while in glanderous horses the test results are 88.8 per cent. positive, 3.5 per cent. negative, and 7.5 per cent. doubtful. As with the complement-fixation test the doubtful and negative reactions occur chiefly in the early stages of the disease; consequently its use alone, without the agglutination reaction, to check the complement-fixation test is subject to error unless retests at suitable intervals are planned and carried out.

Technic of Application.—When 2 or 3 drops of concentrated mallein are instilled into the conjunctival sac, no reaction save a slight lacrimation and congestion results in healthy horses. In glanderous animals this goes on, at the end of from five to seven hours, to profuse lacrimation, redness, edema, and the formation of pus. There may be only a drop of pus at the inner canthus of the eye, or all degrees to profuse purulent discharge. Unless pus is present, the reaction is not considered positive. The results may be recorded according to the following scheme suggested by the Bureau of Animal Industry: N = negative—eye unchanged; S = suspicious—sero-mucous discharge; P+ = positive—sero-mucous discharge with purulent flakes; P++ = positive—distinct purulent discharge; P+++ = purulent discharge with swelling of eye-lids; P++++ = marked purulent discharge with swelling and gluing together of the eye-lids. There is a slight rise in temperature in those cases showing a marked reaction, but as the local reaction is very distinct, the tedious task of taking temperatures as in the subcutaneous method is superfluous.

Another advantage of this method over the subcutaneous inoculation, aside from its simplicity, is the fact that it can be repeated after twenty-four hours in doubtful cases; also it does not interfere with subsequent serodiagnostic tests. With very few exceptions, a second test in a glanderous horse gives a prompt reaction.

The intrapalpebral mallein test is a modification of the ophthalmic-mallein test. Instead of applying the mallein directly to the eye a diluted mixture is injected intradermally into the delicate skin of the lower eyelid. This test gives a local and a thermal reaction, accompanied sometimes by a general reaction.

Directions.—Dilute one volume of eye mallein to two or three volumes by adding a 0.5 per cent. carbolic salt solution just before using. A fold of the thin skin of the lower eyelid is grasped by the thumb and forefinger as near the margin as possible. Then the fine bore-needle of a hypodermic syringe is inserted into the dermis parallel to the margin of the eyelid midway between the inner and outer canthus. The needle should be inserted about half an inch and 0.1 c.c. of the freshly diluted eye mallein injected.

Local Reaction.—In the *normal* horse an inflammatory edema appears at the site of injection reaching its height at about the eighth to tenth hour and then subsides in twenty-four to thirty-six hours. This edema is neither painful nor diffuse and usually takes on a crescentic shape. The conjunctival membrane is not congested although occasionally a slight mucous or serous discharge is present. This discharge differs markedly from that of a horse which has glanders. Thermal reaction: There is no rise in temperature in a normal horse.

In a *glandered horse* the injection is followed in a few hours by a hot, painful, diffuse swelling, which may involve the upper lid and whole side of the upper part of the face—occasionally corded lymphatics, radiating from the swelling, may be noted. The conjunctival membrane is congested and a conjunctivitis with a dirty purulent discharge usually develops. The free edge of the eyelid is swollen and tends to protrude upward at the inner canthus. The height

of the reaction may occur at the eighth to the tenth hour or as late as the forty-eighth or fifty-sixth hour. It may last seven days but usually only four or five days. Thermal reaction: The temperature rise may reach 105° F. and as a rule follows the local reaction in its occurrence. The *general reaction* is not marked but may involve dulness and loss of appetite.

This test has a transient effect on the serodiagnostic tests. One of its advantages, however, is that the test cannot be interfered with by an unscrupulous owner.

Subcutaneous Injection of Mallein.—The injection of mallein subcutaneously is one of the oldest and most reliable methods for the diagnosis of glanders. It should be applied however only after the complement-fixation, agglutination and eye mallein tests have been used, since the subcutaneous injection of mallein, as also any glanders antigen including vaccines, interferes with the serodiagnostic tests. In spite of the fact that it picks up 89 per cent. (Hutyra and Marek[1]) of the positive cases, its cumbersome technic of prolonged temperature taking, the detention of the horses from work, etc., all contribute to render it unsuitable as an early test.

Before applying the subcutaneous test the temperature of the horse should be taken at least three times at intervals of three hours. If there is fever the mallein should not be given. The injection of mallein (usually about 2 c.c.) should be made about 10 P.M. In a glanderous horse there will be a local reaction and a general reaction with fever. The temperature begins to rise usually three or four hours after the injection and reaches its maximum between the tenth and twelfth hours. Sometimes the highest point is not reached until fifteen to eighteen hours after the injection. This rise in temperature is from 1.5° to 2° C. (2° to 3.5° F.). The temperature taking should be continued every two hours, beginning not later than eight to ten hours after the mallein was given. The general condition of the animal is more or less profoundly modified and the local reaction is usually very marked around the point of injection. Here, in a few hours, there appears a warm, tense and very painful swelling. Running from this will be found hot, sensitive lines of sinuous lymphatics directed toward the neighboring lymphatic nodes. This edema increases for twenty-four to thirty-six hours and persists for several days, not disappearing entirely for eight or ten days.

In healthy animals the rise of temperature is usually only a few tenths of a degree but it may reach 1° C. This rise should always be considered, however, in connection with the general and local reactions. At the point of injection the mallein produces only a small edematous tumor which, instead of increasing diminishes rapidly and disappears in about twenty-four hours.

Occurrence of the Reactions of the Various Tests after Infection.—*Agglutinins* increase above normal in four or five days and continue to rise in the early stages of the disease, diminishing as the disease becomes chronic. Specific amboceptors for the *complement-fixation* test may be demonstrated in from seven to ten days and remain during the entire course of the disease. The *subcutaneous mallein* test may, as a rule, be relied upon for diagnosis fifteen days after infection, while the *eye mallein test* is reliable twenty-one days after infection.[2]

[1] Centralb. f. Bact., 1909, Band, 52.
[2] Proc. Fifteenth Meeting, Am. Vet. Assn., 1913, p. 291.

Effect of One Test on the Others.—The serodiagnostic tests are influenced in three to six days after a subcutaneous injection of mallein or any glanders antigen including vaccines. The period of influence varies from six to eight weeks after the injection of mallein, and lasts three months or longer after the injection of glanders antigen or vaccines.

The injection of intrapalpebral mallein has a transient effect only on the serodiagnostic tests.

Postmortem Lesions.—Postmortem lesions are given above. The confirmation of the findings of all positive (or doubtful) tests by careful examinations at autopsy, injection of material into guinea-pigs, together with the study of microscopic sections, is most desirable in order to extend our present data.

Preparation of Mallein.[1]—Mallein for *subcutaneous injection* is produced by growing B. mallei (preferably several strains) for six to eight weeks in a 5 per cent. glycerin-nutrient veal bouillon, 2.5 acid to phenolphthalein about pH 6.4. (See chapter on Media.) Each flask or bottle of the culture is then tested for purity by the examination of smears and cultures made on veal agar *neutral* to phenolphthalein (about pH 8 or 8.2)—a medium unfavorable to the growth of B. mallei. If pure, the broth culture is killed by steaming in the Arnold sterilizer for one hour. After sedimentation in the ice-box for a few days the supernatant fluid is filtered first through paper pulp and then through the Berkefeld filter. Carbolic acid is added to give 0.5 per cent.

For *ophthalmic mallein* the same procedure as above is followed except that after filtering the liquid through paper pulp, it is measured and then evaporated over the hot-water bath to one-tenth its volume. The viscid liquid is then sterilized by heating in the Arnold sterilizer for three-quarters of an hour. The precipitate which has formed is thrown down either by centrifugalizing, or by sedimentation in the ice-box. This latter method takes about two weeks.

Before use both the *subcutaneous* and the *ophthalmic* mallein should be subjected to potency tests on both glandered and normal horses.

[1] The methods given are those in use in the Health Department of the City of New York.

CHAPTER XXX.

THE BACILLUS AND THE BACTERIOLOGY OF TUBERCULOSIS.[1]

Historical Note.—A knowledge of phthisis was certainly present among men at the time from which our earliest medical descriptions come. For over two thousand years many of the clearest thinking physicians have considered it a communicable disease; but it is only within comparatively recent times that the infectiousness of tuberculosis has become an established fact in scientific medicine. Villemin, in 1865, by infecting a series of animals through inoculations with tuberculous tissue, showed that tuberculosis might be induced, and that such tissue carried the exciting agent of the disease. He also noticed the difference in virulence between tuberculous[2] material of human and bovine sources, and said that not one of the rabbits inoculated with human material showed such a rapidly progressive and widespread generalization as those receiving material from the cow. Baumgarten demonstrated, early in 1882, bacilli in tissue sections which are now known to have been tubercle bacilli. But these investigations and those of others at the same time, though paving the way to a better knowledge of the disease, proved to be unsatisfactory and incomplete. The announcement of the discovery of the tubercle bacillus was made by Koch in March, 1882. Along with the announcement satisfactory experimental evidence was presented as to its etiological relation to tuberculosis in man and in susceptible animals, and its principal biological characters were given.

Distribution of Bacilli.—They are found in the sputum of persons and animals suffering from pulmonary or laryngeal tuberculosis, either free or in the interior of pus cells; in miliary tubercles and fresh caseous

[1] For monographs see: Baldwin, Petroff and Gardner: Tuberculosis, Lea & Febiger, 1927. Willis: Laboratory Diagnosis and Experimental Methods in Tuberculosis, Thomas, 1928. Calmette: Tubercle Bacillus Infection and Tuberculosis in Man and Animals, translated by Soper and Smith, Williams & Wilkins, 1923.

[2] Tuberculous—infected by tubercle bacillus; tubercular—presence of tubercles which may develop from other causes.

EXPLANATION OF PLATE VI.

FIG. 1.—Tuberculous lymph node "giant cell" containing tubercle bacilli "human type." Bacilli red, rest of specimen blue. Ziehl-Neelsen stain. × 1000 diam.

FIG. 2.—Tuberculous sputum from human case. Stain same as above. × 1000 diam.

FIG. 3.—Tuberculous sputum, human case. Stained by Hermann's method. Tubercle bacilli violet, rest of specimen brown.

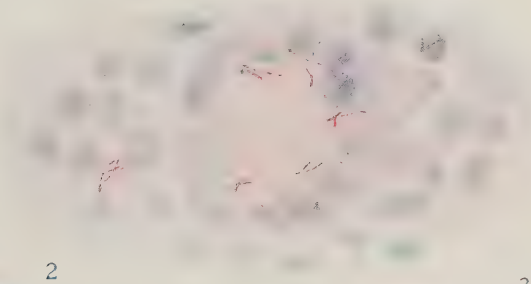
FIG. 4.—Pus from tuberculous abscess in cow. "Bovine type" of bacillus. Stained same as Figs. 1 and 2. × 1000 diam.

FIG. 5.—Section through leprous skin showing bacilli in clumps in and out of cells us dlarge "leprous cell" containing a ball of bacilli. Stained with Ziehl-Neelsen.

FIG. 6.—Photograph of human type of tubercle bacilli from sputum. Bacilli in red, rest of specimen blue. × 1000 diam. (Fränkel and Pfeiffer.)

PLATE VI

1



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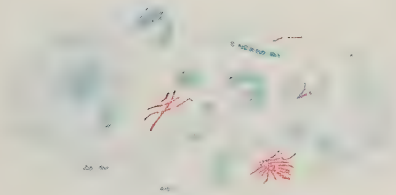

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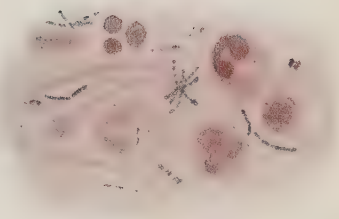

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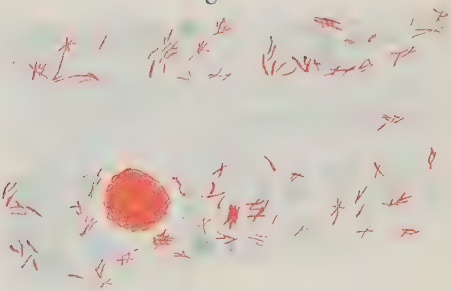

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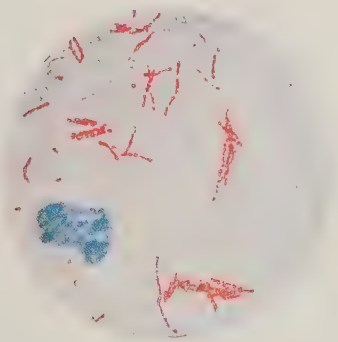


masses in the lungs and elsewhere; in recent tuberculous cavities in the lungs; in tuberculous glands, joints, bones, serous effusions, mucous membranes, and skin affections. They are also found in the feces of those suffering from tuberculosis of the intestines or of those who have swallowed tuberculous sputum. They are frequently present in the blood in very small numbers, in large number only in acute miliary tuberculosis.

Morphology.—The tubercle bacilli (Mycobacterium tuberculosis) are slender, non-motile rods of about 0.3μ in diameter by 1.5μ to 4μ in length. (Plate VI, Figs. 1 and 2.) The morphology is extremely variable, especially on culture media, and varies with the type of medium used. Commonly they occur singly or in pairs and are then usually slightly curved; frequently they are observed in smaller or larger bunches. Under exceptional conditions branched and club-shaped forms are observed. The tubercle bacillus, therefore, seems to be allied to Actinomyces. In stained preparations there are often seen unstained portions. In old cultures irregular forms may develop, the rods being occasionally swollen at one end or presenting lateral projections. Here also spherical granules appear which stain with more difficulty than the rest of the bacillus and also retain the stain with greater tenacity. The bacilli, however, containing these bodies are not appreciably more resistant than those not having them; therefore they cannot be considered true spores. The bacilli have a thin capsule, shown in one way by the fact that they appear thicker when stained with fuchsin than with methylene blue. It is claimed that a filtrable stage of the bacillus exists.[1] It is believed that the filtrable forms are the granules of Müch, Gram-positive granules which may be found in the bacilli or free. We do not believe that there is a filtrable form which continues to reproduce only filtrable particles.

Chemical Constituents of the Tubercle Bacilli.—Water 86 per cent., dry substance 14 per cent., one-fourth of which is soluble in alcohol and ether, consisting of free fatty acids and fatty acids combined with the higher alcohol "mykol" to form a wax; lecithin proteins, other nucleoalbumins, and inorganic bases constitute the remainder. A specific "residue antigen" has been separated from tubercle bacilli, a complex carbohydrate substance.[2]

Staining Peculiarities.—These are very important, for by them its recognition in microscopic preparations of sputum, etc., is rendered possible. Owing to content of waxy substance it does not readily take up the ordinary anilin colors, but when once stained it is very difficult to decolorize, even by the use of strong acids. The more recently formed bacilli are much more easily stained and decolorized than the older forms. For methods of staining see Chapter III

Biology.—The bacillus of tuberculosis is a *parasitic, aërobic, nonmotile* bacillus, and grows best at a temperature of about 37° C., limits 30° to 42° C. It does not form spores.

[1] Centralbl. f. Bakteriol., ref., 1912, **51**, 244; Compt. rend. Soc. de biol., 1927, **96**, 138.
[2] Jour. Immunol., 1926, **12**, 137.

Resistance.—It has been assumed that the bacilli, because of the protection given by their waxy substances, have a somewhat greater resisting power than most other pathogenic bacteria. Frequently a few out of a great number of bacilli resist desiccation at ordinary temperatures for months; most bacilli die, however, soon after drying. There is a greater resistance shown by the tubercle bacillus than by most other non-sporebearing bacilli to the action of the products of bacterial growth as in souring milk, in water, and in sewage. In water and sewage they may remain viable for weeks. They frequently retain their vitality for several weeks, or even months, in putrefying material, such as sputum. In cultures the bacilli do not live longer than three months, unless the media be favorable, such as egg or serum; transplants after this time may fail to grow. A few bacilli, sufficient to infect guinea-pigs, may persist much longer. Cold has little effect upon them. When dry, some of the organisms stand dry heat at 100° C. for twenty minutes but are dead in forty-five minutes; but when in fluids and separated as in milk, they are quickly killed, viz: at 60° C. within fifteen minutes, at 65° C. within two minutes, at 70° C. within thirty seconds, at 85° C. within twenty seconds and at 100° C. within ten seconds. In some experiments they have appeared to withstand a higher temperature. As pointed out by Theobald Smith, when milk is heated in a test-tube in the usual way the cream which rises on heating is exposed on its surface to a lower temperature than the rest of the milk, and as this contains a large percentage of the bacteria some of them are exposed to less heat than those in the rest of the fluid. Rosenau points out another source of error: if a moderate number of killed bacilli is injected into guinea-pigs limited lesions will arise and caseation may follow. On killing and autopsying the animals, tubercle bacilli can then be demonstrated in smears from the lesions, and the inoculation is considered positive. If, however, this material is reinjected into a second pig, the latter will show nothing on autopsy. This capacity of dead bacilli to cause macroscopic lesions was shown by Prudden and Hodenpyl. Its importance, however, is not sufficiently considered.

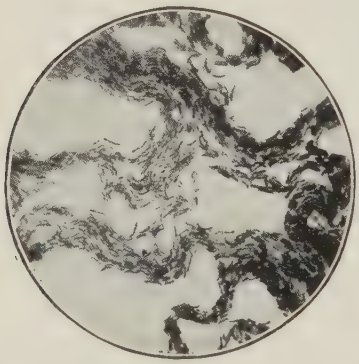


FIG. 147.—Tubercle bacilli. Impression preparation from small colony on coagulated blood serum. × 1000 diameters.

The resisting power of this bacillus to chemical disinfectants, drying and light is considerable, but not as great as it is apt to appear, for, as in sputum, the bacillus is usually protected by mucus or cell protoplasm from penetration by the germicidal agent. It is not always destroyed by the gastric juice in the stomach, as is shown by successful infection experiments in susceptible animals by feeding them with tubercle bacilli.

In sputum it will resist 3 per cent sulphuric acid. They are destroyed in sputum in six hours or less by the addition of an equal quantity of a 5 per cent. solution of carbolic acid. Bichloride of mercury is less suitable for the disinfection of sputum, as it combines with the mucus and forms a more or less protecting envelope. Sulphur and formaldehyde disinfection will kill tubercle bacilli. (See Disinfection.) The tubercle bacillus resists the action of alkaline hypochloride solution ("antiformin") in dilutions which quickly dissolve non-acid-fast bacteria.

The tubercle bacillus in sputum when exposed to direct sunlight is killed in from a few minutes to several hours, according to the thickness of the layer and the season of the year; it is usually destroyed by diffuse daylight in from five to ten days.

Dried sputum in rooms protected from abundant light has occasionally been found to contain virulent tubercle bacilli for as long as ten months. For a year at least it should be considered dangerous. The Röntgen rays have a deleterious effect on tubercle bacilli in cultures, but practically none upon those in tissues.

FIG. 148.—Tubercle bacilli, bovine. × 1000 diameters.

FIG. 149.—Tubercle bacilli, human. × 1000 diameters.

Multiplication of Tubercle Bacilli in Nature Takes Place Only in the Living Animal.—The tubercle bacillus is a strict parasite—that is to say, its biological characters are such that it could scarcely find natural conditions outside of the bodies of living animals favorable for its multiplication. Under exceptional conditions, such as in freshly expectorated sputum, tubercle bacilli may increase for a limited time.

Cultivation of the Tubercle Bacillus.—On account of their slow growth and the special conditions which they require, tubercle bacilli cannot be grown in pure culture by the usual plate method on ordinary culture media. Koch first succeeded in cultivating and isolating this bacillus on coagulated beef serum, which he inoculated by carefully rubbing the surface with sections of tuberculous tissue and then leaving the culture, protected from evaporation, for several weeks in the incubator. Cultures are more readily obtained of human or avian than of bovine bacilli.

Growth on Coagulated Dog or Bovine Serum or on Egg.—The egg medium is generally used to obtain the first culture. The growth on egg or serum media is usually visible at the end of ten days at 37° C., and at the end of three or four weeks a distinct and characteristic development has occurred. On serum small, grayish-white points and scales first appear on the surface of the medium. As development progresses there is formed an irregular, membranous-looking layer. On egg the growth is in the form of more or less elevated colonies which may become confluent. Colony variants on gentian-violet egg medium have been observed by Petroff.[1]

Growth on Nutrient 3 to 5 Per cent. Glycerin Agar.—Owing to the greater facility of preparing and sterilizing *glycerin agar*, it may be more convenient for continuing to produce later cultures. When numerous bacilli have been distributed over the surface of the culture medium, a rather uniform, thick white layer, which subsequently acquires a slight yellowish tint, is developed; when the bacilli sown are few in number, or are associated in scattered groups, separate colonies are developed, which acquire considerable thickness and have more or less irregular outlines. The growth appears similar to that shown upon bouillon as seen in Fig. 150.

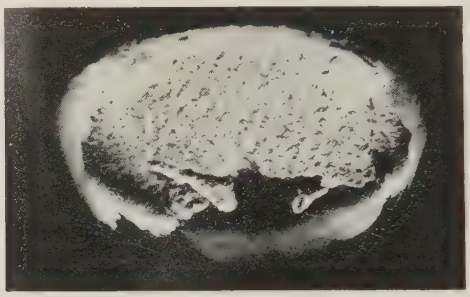


Fig. 150.—Growth of tubercle bacilli upon glycerin bouillon. (Kolle and Wasserman.)

Growth on Nutrient Veal or Beef Broth Containing 5 Per Cent. of Glycerin.—Glycerin broth is used for the development of tuberculin and must be neutral to litmus, viz., between 1.5 and 2 per cent. acid to phenol phthalein. On these media the tubercle bacillus grows readily if a very fresh thin film of growth from the glycerin agar or a small piece of pellic le removed from a previous broth culture is floated on the surface. This continues to enlarge as long as it floats on the surface of the liquid, and in the course of three to six weeks covers it wholly as a single film, which on agitation is easily broken up and then settles to the bottom of the flask, where it ceases to develop further. The liquid remains clear. A practical point of importance, if a quick growth is desired, is to use for the new cultures a portion of the pellicle of a growing bouillon culture which is very thin and actively increasing.

Growth on Potato.—A good growth from cultures and sometimes even from tissue takes place on potato and this forms a very satisfactory medium for stock cultures.

[1] Proc. Soc. Exp. Med. and Biol., 1927, **25**, 14.

Obtaining of Pure Cultures of the Tubercle Bacillus from Sputum, Infected Tissue, and Other Materials.—On account of the time required and the difficulties to be overcome, this is never desirable except when careful investigations of importance are to be undertaken. *Pure cultures* can be obtained directly from tuberculous material, if the tubercle bacilli are present in sufficient number and mixed infection is not present, by using the proper blood serum or egg culture medium (p. 122); but it is difficult to get material free from other bacteria which grow much more rapidly and take possession of the medium before the tubercle bacillus has had time to form visible colonies. It is safest first to inoculate guinea-pigs, subcutaneously or intramuscularly, preferably into the thigh, and then obtain cultures from the animals as soon as the tuberculous infection has developed. In this way, because of the susceptibility of the guinea-pig to tuberculosis, cultures may be obtained from material containing very few tubercle bacilli, although contaminating bacteria may be very numerous.

Animals inoculated usually die at the end of three weeks to four months. It is better, however, not to wait until the death of the animals, but at the end of four to six weeks to kill a guinea-pig without violence, using illuminating gas, chloroform or ether in a closed tin or jar. (Animals which develop tuberculosis acutely are apt to have abundant tubercle bacilli and give successful cultures, while the chronic cases usually have few bacilli and may give unsuccessful cultures.) The animal after being killed is tied out in a tray, and after washing with a 5 per cent. solution of carbolic acid, immediately autopsied. The skin over the anterior portion of the body having been carefully turned back, the inguinal nodes are removed with fresh instruments. The nodes on the side of injection are especially favorable for cultures. The abdomen is then opened and the spleen and retroperitoneal nodes removed. As the organs are removed they should be placed in Petri dishes and thoroughly minced with knife and forceps. Fresh instruments should be used for each operation. The sternal nodes may be used for cultures, but the lungs are almost useless, as the majority of cultures will be contaminated. The minced tissue is then placed on the surface of the culture media, both egg and glycerin-egg being used, and evenly and thoroughly smeared over its surface, then the cotton plug is dipped in hot paraffin to aid in keeping the media from drying. The tubes are incubated in an inclined position. On egg, growth is visible in from seven to ten days and well marked at the end of three weeks. Many tubes should be inoculated, as it is only with the dexterity acquired by practice that contaminations are avoided. As will be noted further on, the growth of the bovine type will be very sparse and on glycerin-egg probably negative.

Cultures may also be obtained with a fair proportion of successful results by the antiformin method, the method of Petroff or of Corper. In the former (see p. 519) the washed sediment is inoculated on egg media. Petroff (see Media) digests sputum or feces with equal amounts of 3 per cent. NaOH for one-half hour at 37° C., neutralizes with dilute HCl and inoculates the sediment obtained by centrifugalizing, on his special gentian-violet egg medium. Corper mixes 1 c.c. of sputum (urine or tissue) with 1 c.c. of 6 per cent. sulphuric acid, incubates at 37° C. for one-half hour, dilutes with about 10 c.c. of saline, centrifugalizes and inoculates the sediment on crystal-violet potato medium. (See Media.) Successful cultures of bacilli in spinal fluids can be obtained

by inoculating the sediment obtained by centrifugalizing. A successful culture of an acid-fast bacillus does not necessarily mean a tubercle bacillus. Van Winkle in our laboratory has isolated two non-pathogenic acid-fast strains from sputa.

Pathogenesis.—The tubercle bacillus is pathogenic not only for man, but for a large number of animals, such as the cow, monkey, pig, cat, etc. Young guinea-pigs are very susceptible, and are used for the detection of tubercle bacilli in suspected material. When inoculated with a minute dose of living bacilli they usually succumb to the disease. Infection is most rapidly produced by intraperitoneal injection. If a large dose is given death follows in from fourteen to twenty days. The omentum is found to be clumped together in sausage-like masses which contain many bacilli. There is no serous fluid in the peritoneal cavity, but generally in both pleural sacs. The spleen is enlarged, and it, as well as the liver and peritoneum, contains large numbers of tubercle bacilli. If smaller doses are given the disease is prolonged. The peritoneum and internal organs—spleen, liver, etc., and often the lungs—are then filled with tubercles. On subcutaneous injection, for instance, in the knee-fold region (commonly called inguinal) there is a thickening of the tissues about the point of inoculation, which may break down in one to three weeks and leave a sluggish ulcer covered with cheesy material. The neighboring lymph nodes are swollen, and at the end of two or three weeks may attain the size of hazel-nuts. Soon an irregular fever is set up and the animal becomes emaciated, usually dying within four to eight weeks. If the injected material contained only a small number of bacilli the wound at the point of inoculation may heal up and death be postponed for a long time. At autopsy the lymphatic nodes are found to have undergone cheesy degeneration; the spleen may be very much enlarged, and throughout its substance, which is colored dark red, are distributed masses of nodules. The liver is also commonly increased in size, streaked brown and yellow, and the lungs may be filled with grayish-white tubercles; but, as a rule, the kidneys contain no nodules. Occasionally the lesions are limited to the knee-fold and retroperitoneal nodes. Tubercle bacilli are found in the affected tissues, but the more chronic the process the fewer the bacilli present.

Injection into the knee-fold regions is to be preferred for diagnostic purposes, the swelling of the local lymph nodes being then palpable. As soon as this is appreciable the node may be removed with or without killing the pig, the presence or absence of tuberculous lesions noted and smears made for the detection of tubercle bacilli, thus saving time. It must be remembered that the pig may not show the usual picture of generalized tuberculosis, but only a swelling of the local lymph nodes. Fortunately tubercle bacilli are usually demonstrable in smears made from the crushed nodes. If there is any doubt the remaining tissue should be emulsified and reinjected into a second set of pigs. Another point to be considered is that rarely other organisms may give a picture difficult to distinguish macroscopically from tuberculosis, as, for instance, streptothrix. To safeguard against error smears

should be stained and tubercle bacilli demonstrated. Chronic guinea-pig septicemia may be accompanied by lesions in the spleen which might be taken for tuberculous lesions.

Rabbits are very susceptible to tuberculosis of the bovine type, less so to that of the human type. This will be given more in detail under the differences between human and bovine tuberculosis.

Monkeys are very susceptible to infection with both types of bacilli. Cats, dogs, rats and mice are susceptible, the last two usually show no tuberculous lesions, but there is great multiplication of the bacilli in the tissues.

Tubercle Toxins.—No true toxins have been demonstrated. Eberson[1] has shown that a heat-labile, toxic substance is present in broth cultures which is neutralizable by an anti-serum. This toxic substance gives skin reactions in arrested or moderately advanced cases of tuberculous infection, not in advanced cases. In this regard the substance seems different from the extracellular toxins of other bacteria. The bodies of the bacteria contain substances which cause necrosis of tissue with subsequent caseation or abscess. In broth cultures, after filtration, are present substances which produce fever and inflammatory reactions of tissues. These substances as well as extracts from tubercle bacilli are highly toxic for tuberculous animals, little if at all for normal animals and cause fever only in the former and the tissue inflammation spoken of as marked about tuberculous lesions. These poisons will be considered in detail later in connection with tuberculins.

Action upon the Tissues of the Poisons Produced by the Tubercle Bacillus.—Soon after the introduction into the tissues of tubercle bacilli, either living or dead, the cells surrounding them begin to show that some irritant is acting upon them. The connective-tissue cells become swollen and undergo mitotic division, the resultant cells being distinguished by their large size and pale nuclei. A small focus of proliferated epithelioid cells is thus formed about the bacilli, and according to the intensity of the inflammation these cells are surrounded by a larger or smaller number of lymphoid cells. When living bacilli are present and multiplying the lesions progress, the central cells degenerate and die, and a cheesy mass results, which later may lead to the formation of cavities. Dead bacilli, on the other hand, unless bunched together give off sufficient poison to cause the less marked changes only (Prudden and Hodenpyl). Of the gross pathological lesions produced in man by the tubercle bacilli the most characteristic are small nodules, called miliary tubercles. When young, and before they have undergone degeneration, these tubercles are gray and translucent in color, somewhat smaller than a millet-seed in size and hard in consistence. But miliary tubercles are not the sole tuberculous products. The tubercle bacilli may cause diffuse growth of tissue identical in structure with that of miliary tubercles, that is, composed of a basement substance, containing epithelioid, giant and lymphoid cells. This diffuse tuberculous tissue also tends to undergo cheesy degeneration.

[1] Jour. Am. Med. Assn., 1928, **90**, 260.

Point of Entrance of Infection.—Infection by the tubercle bacillus takes place usually through the respiratory tract or the digestive tract, including the pharynx and tonsils, more rarely through wounds of the skin.

Tuberculosis may be considered to be caused chiefly by the direct transmission of tubercle bacilli to the mouth through soiled hands, lips, handkerchiefs, milk, etc., or by the inhalation of fine particles of mucus thrown off by coughing or loud speaking, or of dust contaminated by tuberculous sputum or feces.

Tuberculosis of Skin and Mucous Membranes.—When the skin or mucous membranes are superficially infected through wounds there may develop lupus, ulceration or a nodular growth. The latter two forms of infection are apt after an interval to cause the involvement of the nearest lymphatic nodes.

Tuberculosis of Respiratory Tract.—The lungs are the most frequent location of clinically recognizable tuberculous inflammation. On account of their location they are greatly protected from external infection. Most of the bacilli are caught upon the nasal or pharyngeal mucous membranes. Only a small percentage can find their way to the larynx and trachea and still less to the smaller bronchioles. From the examination of the lungs of miners as well as from experimental tests there is no doubt that some of the bacilli may find their way into the deeper bronchi. On the other hand, the lungs are the most likely point of localization of tubercle bacilli which find their way into the blood stream. It is in this way that the lungs frequently become infected. It is now well established that infection taking place through the intestine may find its way by the blood to the lungs and excite there the most extensive lesions with or without leaving any trace of its point of entrance. Even if infection of the lung is slight or entirely absent the tubercle bacilli then find their way to the bronchial nodes, where lesions may develop. Lesions, either active or latent, anywhere in the body may be the source of a subsequent pulmonary infection. Should bacilli find their way into the blood, infection of the lung tissue will result if the resistance is lowered for any reason. The nasal cavities are rarely affected with tuberculosis, but more often the retropharyngeal tissue. Tuberculosis of this tissue as well as that of the tonsils is apt to give rise to infection of the lymph nodes of the neck. It is believed that just as bacilli may pass through the intestinal walls to infect the mesenteric nodes, so bacilli may, without leaving any trace, pass through the tonsils to the nodes of the neck.

Primary infection of the larynx is rare. Secondary infection is fairly common. The region of the vocal cords and the interarytenoid spaces are the special sites attacked.

Infection by Inhalation of Dried and Moist Bacilli.—A rather common mode of infection is by means of tuberculous sputum, which, being coughed up by consumptives, is either disseminated as a fine spray and so inhaled, or, carelessly expectorated, dried and, broken up by tramping over it, sweeping, etc., distributes numerous virulent bacilli in the

dust. As long as the sputum remains moist there is no danger of infection, except by direct contact; it is when it becomes dry, as on handkerchiefs, bedclothes and the floor, etc., that the dust is a source of danger.

A great number of the expectorated and dried tubercle bacilli undoubtedly die, especially, as we have said, when acted upon by direct sunlight; but when it is considered that as many as five billion virulent tubercle bacilli may be expectorated by a single tuberculous individual in twenty-four hours, it is evident that even a much smaller proportion than are known to stay alive will suffice in the immediate vicinity of consumptives to produce infection unless precautions are taken to prevent it. The danger of infection is greatest, of course, in the close neighborhood of tuberculous patients who expectorate profusely and indiscriminately, that is, without taking the necessary means for preventing infection. We found that of 100 tuberculous men admitted to one of the consumption hospitals, only 20 claimed to have taken any care to prevent the contamination of their surroundings by their sputum. There is much less danger of infection at a distance, as in the streets for instance, where the tubercle bacilli have become so diluted that they are less to be feared. In rooms the sputum is not only protected from the direct sunlight, but it is constantly broken up and blown about by the walking, closing of doors, etc.

Exhaustive experiments made by many observers have shown that particles of dust collected from the immediate neighborhood of consumptives, when inoculated into guinea-pigs, produce tuberculosis in a considerable percentage of them; whereas, the dust from rooms inhabited by healthy persons or dust of the streets does so only in an extremely small percentage. Flügge is probably right in thinking that the dust which is fine enough to remain for a long time in suspension in the air is usually free from living bacilli. It is in the coarser though still minute particles, those in which the bacilli are protected by an envelope of mucus, that the germs resist drying for considerable periods. It may be said that the danger of infection from slight contact with the tuberculous is not so great as it is considered by many, but that on this account it is all the more to be guarded against in the immediate neighborhood of consumptives. Those who are most liable to infection from this source are young children; the adult members of the family, the nurses and other associates of persons suffering from the disease are relatively very less likely to become infected, not only because adults are somewhat more resistant, but because many of them are somewhat immune because of previous arrested infection. In this connection, also, attention may be drawn to the fact that rooms which have been recently occupied by consumptives are not infrequently the means of producing infection (as has been clinically and experimentally demonstrated) from the deposition of tuberculous dust on furniture, walls, floors, etc. The danger is not apt to last beyond three months. Flügge has drawn attention to the fact that in coughing, sneezing, loud talking, etc., very fine particles of throat secretion containing bacilli are thrown out and carried by air currents many feet

from the patient and remain suspended in the air for a considerable time. To encourage us, however, we now have a mass of facts which go to show that when the sputum is carefully looked after there is very little danger of infecting others except by close personal contact.

Tuberculosis of Digestive Tract.—Tuberculosis of the gums, cheeks or tongue is rare. The tonsils and pharynx are somewhat more often involved. The stomach and esophagus are almost never attacked. The small intestines are rather frequently the seat of infection from bacilli swallowed with the food or dust-infected mucus. In a striking case four previously healthy children died within a short period of one another. Their nurse was found to have tuberculosis of the antrum of Highmore, with a fistulous opening into the mouth. She had the habit of putting the spoon with which she fed the children into her mouth so as to taste the food before it was given to them.

As already noted, the bacilli frequently pass through the mucous membrane of the gastro-intestinal canal to the lymph glands without leaving any lesions.

Infection by Ingestion of Milk and Milk Products.—Milk serves as a conveyer of infection, whether it be the milk of tuberculous mothers or the milk of tuberculous cows. In this case evidence of infection is usually shown in the mesenteric and cervical lymph nodes or generalized tuberculosis may be caused, while the intestinal walls are frequently not affected. Bacilli accompanied by fat pass much more readily through the intestinal mucous membrane or that of the tonsils and pharynx. The transmission of tubercle bacilli by the milk of tuberculous cows has been abundantly proved.

Formerly it was thought that in order to produce infection by milk there must be a local tuberculous affection of the udder; but it is now known that tubercle bacilli may be found in the milk in small numbers, when adjacent tissue is infected and when careful search fails to detect any udder disease. Schröder has shown that the feces are a very dangerous factor in the dissemination of tubercle bacilli. He compares feces in cattle to sputum in man, since the tubercle bacilli are swallowed by cattle and are to a great extent passed through the intestinal tract without destruction. Rabinowitsch, Kempner and Mohler also proved beyond question that not only the milk of tuberculous cattle, which showed no appreciable udder disease, but also those in which tuberculosis was only detected through tuberculin, frequently contained tubercle bacilli. Different observers have found tubercle bacilli in 10 to 30 per cent. of the samples of unheated milk. Butter may contain tubercle bacilli in higher percentages of samples examined. The milk from cattle suffering from udder tuberculosis usually contains a few hundred bacilli per cubic centimeter, but may contain many millions.

A less probable source of infection by way of the intestines is the flesh of tuberculous cattle. Here the danger is much less, from the fact that meat is usually cooked, and also because the muscular tissues are seldom attacked. In view of the finding of the bovine type of bacilli in a considerable percentage of the cases of tuberculous children tested,

the legislative control and inspection of cattle and milk is an absolute necessity. As a practical and simple method of preventing infection from suspected milk, sufficient heating of the milk used as food must commend itself to all. Human tubercle bacilli may be found in milk as instanced by one sample out of a series of city milks examined in this laboratory by Hess.

Bovine Infection in Man.—Numerous investigations have been made on this point. To Ravenel we believe belongs the credit of isolating the first bovine bacillus from a child. The following tables summarizing the results of a large series of cases give a fair idea of incidence of such infection. As will be seen, children are especially the ones infected, and usually the point of entry is clearly alimentary, as shown by the lesions. Cervical adenitis and abdominal tuberculosis are the most frequent types of infection. Generalized tuberculosis due to bovine infection is less frequent. Bone and joint tuberculosis is usually of the human type. The meninges are less commonly affected by the bovine type than by the human type. Infection of adults is uncommon, though a very small number of cases of pulmonary tuberculosis due to the bovine type of bacillus has been reported.

A careful study of all the factors leads us to estimate that of children drinking the average raw milk supply about 10 per cent. of all deaths caused by tuberculosis under five is due to bovine infection.

After pasteurization of milk had been well established in New York City we examined 36 tuberculous cervical lymph nodes and only 6 were found to contain bovine bacilli. In 1 the beginning of infection was noted before pasteurization was enforced, 2 cases were old enough to have been infected before pasteurization and 3 had used raw milk.

The tables on pages 504 and 505 give a summary of the results obtained in several of the larger investigations. Some English reports have shown a very high percentage of bovine infection, thus Mitchell found that 90 per cent. of cervical lymph-node infection under five years were due to bovine infection. This high incidence among Scottish children is due to the very high percentage of tuberculous cattle and the general practice of feeding raw milk to children.

Transmissibility of Tubercle Bacillus to the Fetus.—The transmission of tubercle bacilli from the mother to the fetus occurs occasionally. Some 30 cases have been recorded of tuberculosis in newly-born infants, and about a dozen cases of placental tuberculosis. As to the infection of the fetus from the paternal side, where the father has tuberculosis of the scrotum or seminal vessels, we have no reason to suppose that such can occur.

Attenuation.—Tubercle bacilli when subjected to deleterious influences slowly decrease in virulence. Some strains lose their virulence when artificially cultivated for some time; some quickly, some very slowly. The strains from lupus are likely to have a low virulence.

Mixed Infection.—In regions where tuberculous processes are on the surface, such as skin infections, and also when the infection itself is multiple, as in diseases of the glands of the neck from tonsillar absorp-

TABLE II.—THE RELATIVE PROPORTION OF HUMAN AND BOVINE TUBERCLE BACILLI INFECTIONS IN A LARGE SERIES OF UNSELECTED CASES[1] EXAMINED AT THE RESEARCH LABORATORY.

Diagnosis of cases examined.	Adults sixteen years and over.		Children five to sixteen years.		Children under five years.		Notes.
	Human.	Bovine.	Human.	Bovine.	Human.	Bovine.	
Pulmonary tuberculosis	281	—	8	—	7	—	Clinical diagnosis only known and therefore no positive details as to the extent of lesions elsewhere.
Tuberculous adenitis, inguinal and axillary	1	—	4	—	—	—	See next.
Tuberculous adenitis, cervical	9	—	19		6	13	In two cases cultures were from axillary nodes but the primary focus was cervical. Another case died shortly afterward with pulmonary tuberculosis.
Abdominal tuberculosis	1	—	1	1	1	3	Milk supply of one child subsequently examined. Tubercle bacilli isolated.
Generalized tuberculosis, alimentary origin	—	—	—	—	1	2	Only three cases given under this heading. Many of the cases in the following subdivisions showed marked intestinal lesions and some possibly were of alimentary origin.
Generalized tuberculosis	2	—	1	—	18	4	One bovine case had tuberculous osteomyelitis of the metatarsal bone.
Generalized tuberculosis including meninges	1	—	—	—	25	1	
Tuberculous meningitis	1	—	2	—	26	2	No autopsy. Extent of lesions elsewhere unknown.
Tuberculosis of bones and joints	1	—	10	—	7	—	
Genito-urinary tuberculosis	6	1	1	—	—	—	The adult bovine case was tuberculosis of kidney Removal of kidney Complete recovery.
Tuberculosis of skin	1	—	—	—	—	—	
Tuberculous abscess	1	—	—	—	—	—	Possibly primary in bone.
Totals	305	1	46	9	91	25	

Double infection in one case. Both types isolated. Generalized tuberculosis including meninges, thirteen months. Mesenteric nodes gave human type. Meningeal fluid gave bovine type.

Total cases, 478.

[1] Unselected cases from the hospitals of New York City. For full résumé and discussion of results see Park and Krumwiede: Jour. Med. Res., vols. **23**, **25** and **27**. Also Park: Am. Rev. Tuberc., 1927, **15**, 399.

tion, there are frequently associated with the tubercle bacilli one or more other varieties of organisms. Those of most importance are the streptococcus, pneumococcus and influenza bacilli. The influence of both the local and systemic effects are undoubtedly unfavorable.

TABLE II.—TABULATION OF TOTAL CASES REPORTED UP TO 1914. (WANG.)[1]

Classification.	Adults, 16 years or over.		Children, 5 to 16 years.		Children under 5 years.	
	Human.	Bovine.	Human.	Bovine.	Human.	Bovine.
Pulmonary tuberculosis, including sputum	1000	5	28	..	45	1
Abdominal tuberculosis	24	7	13	17	29	34
Generalized tuberculosis	39	2	32	3	169	22
Genito-urinary	35	4	4			
Tuberculous meningitis	6	..	13	5	55	10
Skin	12	3	4	6	2	
Cervical adenitis	62	10	61	76	18	75
Axillary adenitis	6	..	8	..	4	
Bone and joint	82	4	255	61	89	54
Latent tuberculosis	2	1	2	2	4	1
Miscellaneous (other forms)	5	2	..	1	..	2
	1273	38	420	171	415	199
Percentage of bovine infection at each age period	2.9		28.9		32.4	

Total in 2516 of 16 per cent. bovine type.

TABLE III.—PROPORTION OF BOVINE INFECTION IN VARIOUS KINDS OF HUMAN TUBERCULOSIS AT ALL AGES IN ENGLAND AND WALES (COBBETT, 1922).

Method of infection.	Varieties of tuberculosis.	Types of tubercle bacilli.	
		Human.	Bovine.
Group I: Aërial infection	Pulmonary	98.7	1.3[2]
	Bronchial glands	94.5	5.5
Group II: Food	Intestinal, peritoneal, mesenteric gland	50.0	50.0
	Cervical gland	54.0	46.0[3]
Group III: Various degrees of dissemination from either aërial or food infection	Generalized tuberculosis	84.0	16.0
	Meningitis	82.0	18.0
	Bones and joints	80.0	20.0[4]
	Genito-urinary	82.0	18.0
Group IV: Skin infection	Lupus	50.0	50.0

[1] Jour. Path. and Bacteriol., 1916, **21**, 131.

[2] If we take the figures available for the whole world the percentage of bovine infection in pulmonary tuberculosis is only 0.4.

[3] Mitchell gives 90 per cent. for Scottish cases; Griffith, 71.4 per cent. for similar cases.

[4] Fraser gives 58.6 per cent. for Scottish cases; Griffith, 29.6 per cent. for similar cases, and in the United States the percentage is 21.6.

In regard to pulmonary tuberculosis, it should be remembered that Baldwin has shown that caseation, ulceration and cavity formation may be produced experimentally in the lungs of animals by the tubercle bacillus alone. Further, it has been found that fever, emaciation and other characteristic symptoms of tuberculosis may be caused by the tubercle bacillus independent of any other associated microörganisms. The preponderance of the evidence supports the view that the lesions

and symptoms in this type of infection may be caused by the tubercle bacillus alone, but not infrequently secondary organisms may contribute to the more severe symptoms or may be largely responsible for the unfavorable progress of the disease.

Individual Susceptibility.—It was believed by many that in demonstrating that tuberculosis was due to a specific bacillus its occurrence was sufficiently explained; but they left out another important factor in the production of disease—individual susceptibility. That this susceptibility, or "predisposition," as it is improperly called, may be either inherited or acquired is now an accepted fact in medicine. At first the inherited susceptibility was considered more important than the acquired, but now much that was attributed to the former is known to be explained by the fact of living in an infected area. The acquired susceptibility may arise from faulty physical development or from depression, sickness, overwork, excessive use of alcohol, etc. Unquestionably, vast differences exist in different individuals in the intensity of the tuberculous process in the lung. That this does not depend chiefly upon a difference in virulence of the infection is evident from the fact that individuals contracting tuberculosis from the same source are attacked with different severity. The possibility of favorably influencing many cases of an existing tuberculosis by treatment also proves that, under natural conditions, there is a varying susceptibility to the disease. Clinical experience teaches, likewise, that good hygienic conditions, pure air, good food, freedom from care, etc., increase resistance and are aids to recovery. Animal experiments have shown that not only are there differences of susceptibility in various animal species, but also an individual susceptibility in the same species. The doctrine of individual susceptibility, therefore, is seen to be founded on fact, although the reasons for it are only partially understood. Certain infectious diseases reduce the resistance to tuberculous infection, the most noteworthy example being measles.

Tuberculosis Immunity.—As in other infectious diseases, various attempts have been made to produce an artificial resistance against tuberculosis. The expectation that the immunological mechanism operating in other bacterial diseases would also be found in tuberculosis has not been realized. As a consequence, it is believed the usual manifestations of humoral immunity have a secondary importance in this disease and that we must ascribe the greater rôle to the tuberculous foci. Tuberculous infection presents certain phases which find no close analogy in most other infectious processes. An infection by the tubercle bacillus may take place and foci of considerable size may develop without the production of sufficient disturbance to the bodily welfare to attract attention. Further, while a frank tuberculous disease may come to a clinical cure, yet the focus commonly remains, and, even though completely walled off, may harbor virulent tubercle bacilli during the whole of the individual's normal life. It has now become established that a latent tuberculous focus present in the body, containing as it does bacillary substance, exerts an appreciable influence on the behavior of

the body to subsequent infection. In the consideration of tuberculosis immunity, therefore, we must carefully bear in mind the distinction between tuberculous infection and tuberculous disease.

Koch was the first to discover that animals already infected with living bacilli reacted differently to an injection of tubercle bacilli than did normal animals. When virulent tubercle bacilli are injected into a healthy animal, tubercles develop at or near the point of inoculation and the infection then progresses and the tubercle bacilli are carried to the spleen, liver, lungs and the intermediate glands with the formation of foci in these various organs. As a consequence, the animal finally succumbs to generalized tuberculosis. In the case, however, of an animal already tuberculous, the inoculation with virulent tubercle bacilli is followed by a quite different sequence of events. Shortly after such an inoculation there is a marked inflammatory reaction at the point of injection, followed by necrosis and possibly sloughing but with no advance of the infection beyond the point of inoculation. Römer has given further details of this phenomenon. He found that if a small dose of tubercle bacilli be given at the second inoculation the local reaction soon subsides and healing results. But, on the other hand, if a large quantity of tubercle bacilli be injected the local process goes on to necrotic sloughing and the animal soon dies of cachexia. The tuberculous animal, therefore, when subjected to an injection of tubercle bacilli is not truly infected but suffers from an intoxication, the degree of which depends upon the quantity of tubercle bacilli introduced. Other studies in tuberculosis emphasize the fundamental importance of Koch's and Römer's observations. By means of the various tuberculin reactions and by the most careful postmortem studies on a great number of individuals, we now know that a majority of human beings are infected with the tubercle bacillus and develop demonstrable tuberculous lesions before they reach the age of eighteen. A few children thus infected may succumb to the disease, but the greater number show no conspicuous signs of the infection, and without the tuberculin reaction or the opportunity of examination after death, the presence of tuberculous infection would pass unsuspected and undetected.

It seems quite probable that the majority of cases of pulmonary tuberculosis developing after the eighteenth year of age are caused not by infection from without but by the dissemination of bacilli from an encapsulated focus acquired and arrested during childhood. The greater morbidity during the age group of about twenty to thirty may be accounted for by the fact that this is usually the period of greatest physical and mental strain and stress, and that it is this stress which breaks down the body's resistance and transforms a latent infection into active disease.

Although it is evident that the presence of a latent focus protects the individual to a marked degree against a subsequent infection by the tubercle bacillus, it is equally apparent that an exacerbation suffered by arrested cases show that the protection acqu red is at best only a relative one. We must therefore conclude that there exists in the

human race no absolute immunity to tuberculosis and that such increased resistance as we may possess is gained at the hazard of an early infection. Also tuberculin hypersensitiveness is an index of the resistance, acquired through infection, to reinfection from without.

In the attempts to establish an active immunity a great number of preparations have been tried. Of these, the tuberculins and vaccines of living bacilli will be discussed.

Tuberculin.—The tuberculins have not fulfilled the hopes that Koch had for them. However, the diagnostic use of tuberculin is of very great value.

Tuberculin is only slightly toxic to the non-tuberculous body, and the characteristic reaction following its application to the tuberculous body must be looked upon as an allergic phenomenon, the allergy of infection. Its manifestations are threefold: there is a local reaction at the point of application, a focal reaction at the site of infection and a general constitutional reaction. The local reaction appears as inflammation in the skin or on the mucous membranes, depending upon the point of application, and it is this phase of the reaction which has been so profitably utilized for the diagnosis of tuberculous infection. The focal reaction consists in vascular changes at the tuberculous lesion. There is a hyperemia with a consequent softening of the focus and a liberation of antigenic or toxic focal products. Krause has shown that these products are inherently toxic and their liberation into the blood stream may occasion the fever, the malaise and the other symptoms characteristic of tuberculin intoxication. It is likely that in addition to this the antigen of the tuberculin may react with such immune bodies as may be present in the infected, and therefore hypersensitive, body and thus contribute to the general reaction.

If the dose of tuberculin is not too great the hyperemia at the focus is transient and the body responds with an increased cellular activity at the focus, resulting, under favorable conditions, in a further proliferation of connective tissue and a more complete encapsulation of the tuberculous lesion. Should the dose of tuberculin, on the other hand, be excessive or the bodily resistance be deficient the focal reaction may lead to a softening and breaking down of the lesion with consequent bleeding, dissemination of the liberated tubercle bacilli and extension of the lesion. Inasmuch as an internal location of the lesion precludes an opportunity for observing the focal reaction, we must look to the general constitutional manifestations as an index of the degree of reaction produced. When tuberculin is used as a therapeutic agent the object is the stimulation of the focus by doses of tuberculin so graded and adjusted to the sensitiveness of the individual that the body's ability to respond to and control the focal hyperemia is not overtaxed. In this way cell proliferation, encapsulation and ultimate healing of the focus may be promoted.

The Chemical Nature of Tuberculin.—Koch believed the active principle to be protein in nature. Others studied the subject, but it remained for Long and Seibert to obtain finally the active principle in

a purified state. They found that the growth of the tubercle bacillus on non-protein fluid media resulted in the production of tuberculin but protein accumulated in the media at the same time. By chemical separation methods and by digestion processes they conclude that the activity of tuberculin appears definitely associated with a protein substance. Seibert[1] has obtained this protein in crystalline form.

Tuberculin in Diagnosis and Therapy.—From the foregoing it can be readily seen that by taking advantage of the allergic or hypersensitive state in tuberculosis we are able to use tuberculin both as a diagnostic aid and as a therapeutic agent. *Tuberculin should never be sprayed out of a syringe. It may cause serious reactions in those exposed.*

As a Diagnostic Aid.—The presence of a tuberculous focus in the body, by some mechanism not yet wholly understood, sensitizes the skin and mucous membranes in such a way that the application of tuberculin to these tissues causes a more or less severe inflammatory reaction at the point of application. The preparation used is the Old Tuberculin ("O. T.") of Koch and the following represents the methods of application.

The Intracutaneous Test of Mantoux.—While slightly more difficult to carry out than the cutaneous test, the intracutaneous is the more delicate. C. H. Smith finds the test about twice as delicate as the von Pirquet cutaneous method. The total volume injected should be 0.1 c.c., and it has been customary to employ several dilutions of tuberculin in order to determine the degree of hypersensitiveness of the patient's skin. For routine exclusion purposes 0.1 c.c. of tuberculin diluted 1 to 1000 is reliable. Children over two years of age are injected with 0.1 c.c., under two years with one-half this amount. We distribute a test outfit devised by Krumwiede which contains a capillary tube containing 0.01 c.c. of tuberculin and a bottle containing 10 c.c. of saline, so that the clinician can easily prepare a fresh dilution for the test. The reaction when positive appears in six to eight hours, reaches its maximum in twenty-four to forty-eight hours and generally subsides in six to ten days, and consists of infiltration, hyperemia and, in severe reactions, vesiculation. The width of the area of infiltration and the degree of inflammation are noted. It not infrequently happens that a person fails to give a positive reaction at the first test yet shows the typical local manifestations when the test is repeated. This has given rise to the impression that an injection of tuberculin sensitizes the individual. Without further discussion it may be stated that, as far as we know, there is no skin sensitiveness without infection. The appearance of a positive reaction at the second injection may be looked upon as a true reaction, and it is likely that the first injection, while eliciting no response in the skin, has served to stimulate the latent hypersensitiveness of the cells.

The Cutaneous Test of von Pirquet.—Two small similar scarifications or scratches are made about 3 inches apart. Oozing of blood is to be

[1] Am. Rev. Tuberc., 1928, **17**, 402.

avoided. On one spot or scratch a drop of tuberculin is placed and allowed to dry on the scarification. The tuberculin may be diluted to 25 per cent. if desired. The other spot is kept as a control. Both spots should be examined at the end of twelve, twenty-four and thirty-six hours. A positive reaction appears after three to twenty-four hours and is usually at its height at thirty-six to forty-eight hours, and consists then of slightly raised reddening of the skin somewhat circular in outline and usually about 10 mm. in diameter. Reactions under 5 mm. in diameter should be regarded as doubtful.

The Subcutaneous Test.—The object of this test is to elicit a constitutional reaction in infected persons by the injection of old tuberculin under the skin. Owing to the severe reactions frequently obtained which resu ted in harm to the patient it is now considered the better practice to abandon this test in favor of the Mantoux method. Its tendency to increase the inflammatory reaction in the infected area is sometimes of value in diagnosis. On the other hand, deliberately to cause a focal reaction by increasing the dose of tuberculin is generally considered an undesirable practice.

The Percutaneous Test of Moro.—An ointment is made of equal parts of lanolin and tuberculin ("O. T."), and a small amount of it is rubbed into the skin on the chest. A positive reaction is shown by the development of reddening and papules.

Ophthalmic Test of Calmette.—Owing to the occurrence of serious accidents this test is little used. A drop of a 2 per cent. solution of tuberculin is applied to the lower conjunctival sac. The reaction is indicated by secretion and reddening of the inner canthus carbuncle or lower lid, which may include the entire conjunctiva, with edema of the lids.

Deductions and Limitations of the Tuberculin Test for Diagnosis.—A positive reaction indicates the presence of a tuberculous focus but not necessarily of tuberculous disease. It tells nothing of the extent or activity of the lesion. Krause gives the following basis for interpreting the reaction: There is no cutaneous hypersensitiveness without a focus (tubercle); this hypersensitiveness appears coincident with the establishment of the focus; it diminishes with the healing of the focus; it varies directly with the intensity of the disease; however, many advanced cases, particularly those in cachexia, fail to show any appreciable response to tuberculin tests. Measles diminishes hypersensitiveness.

Tuberculin: Kinds and Preparation.—There exist a large number of tuberculin preparations. The following, however, hold the highest favor and suffice for the needs of the diagnostician or practitioner:

Tuberculin, Koch's "Old" (O. T.).—Cultures of tubercle bacilli after six weeks' growth on 5 per cent. glycerin broth[1] are heated in the Arnold sterilizer to kill the bacilli and filtered. This bacillus-free filtrate is evaporated to one-tenth its original bulk and after filtering through paper to remove the sediment it is ready for use. The tuberculin is, therefore, a heated 50 per cent. glycerin solution of the products of the bacilli in the culture fluid and such portions of

[1] Broth should be made 1.5 per cent. acid to phenolphthalein.

the bacilli as go into solution. As this tuberculin is used for diagnostic work it should be tested for potency. This may be done by determining: (*a*) The killing dose for tuberculous guinea-pigs; (*b*) the skin-reacting dose in such pigs; (*c*) titration, using the skin of a tuberculous human; (*d*) the intratesticular injection of tuberculous guinea-pigs.[1]

Tuberculin B. F. (Bouillon Filtrate of Denys) is made from a broth culture as above. The culture is not heated, but filtered first through paper and then through a Berkefeld filter to insure sterility. It differs from the original tuberculin, in that it is neither heated nor concentrated, and contains only such constituents of the bacillus as are soluble or are developed in the culture medium during cultivation.

Tuberculin B. E. (Bacillus Emulsion) is produced by grinding up dried tubercle bacilli until no intact bacilli can be found on microscopic examination. This powder is then suspended in glycerin-water and heated to 60° C. for an hour or more to kill any viable tubercle bacilli. The proportion of bacillus to water is such that 1 c.c. of fluid contains 5 mg. of bacillus substance. This preparation contains all the constituents of the tubercle bacillus in an unchanged state and therefore corresponds to a bacterial vaccine.

The undiluted products keep for a long time if kept cool and protected from the light. The low dilutions keep for at least one month, but the high dilutions should not be used after two weeks.

Method of Diluting Tuberculin.—The following describes a method of dilution in terms of volume of finished tuberculin and gives the weight equivalent. Although in the case of bouillon filtrate many use dilutions, taking into consideration the fact that the bouillon filtrate is not concentrated, or in the case of bacillus emulsion dilute according to the weight of solid substance contained, a uniform method for each seems advisable for reasons of simplicity.

If we consider the finished product in terms of cubic centimeters or grams regardless of the contents, the following is the method of dilution. Dilutions should be made with sterile saline to which 0.25 per cent. carbolic has been added.

Dilutions.	Amount of tuberculin.	Amount of diluent.	Content of tuberculin terms of finished product.	Using bacillus emulsion content of solid substance in each dilution will be:
A	1 c.c.	9 c.c.	1 c.c. = 0.1 c.c. or gm., or 100 c.mm. or mg.	1 c.c. = 0.5 mgm.
B	1 c.c. of dilution A	9 c.c.	1 c.c. = 0.01 c.c. or gm., or 10 c.mm. or mg.	1 c.c. = 0.05 mgm.
C	1 c.c. of dilution B	9 c.c.	1 c.c. = 0.001 c.c. or gm., or 1 c.mm. or mg.	1 c.c. = 0.005 mgm.
D	1 c.c. of dilution C	9 c.c.	1 c.c. = 0.0001 c.c. or gm., or 0.1 c.mm. or mgm.	1 c.c. = 0.0005 mgm.
E	1 c.c. of dilution D	9 c.c.	1 c.c. = 0.00001 c.c. or gm., or 0.01 c.mm. or mgm.	1 c.c. = 0.00005 mgm,
F	1 c.c. of dilution E	9 c.c.	1 c.c. = 0.000001 c.c. or gm., or 0.001 c.mm. or mgm.	1 c.c. = 0.000005 mgm.

Tuberculin Treatment.—Tuberculin is not a cure for tuberculosis. It should be used as an addition to, not as a substitute for, the recognized methods of treatment. It is a two-edged weapon and should be employed only by those who have a thorough understanding of its possibilities for good and unfortunately for harm.

The principle of the method is to start with the injection of minute doses one-tenth to one one-thousandth of a milligram and gradually increase the dose by 5 to 10 per cent. additions till the limits of tolerance are reached. The increase is so gradual that the course of treatment

[1] Long: Jour. Inf. Dis., 1925, **37**, 368.

takes a long time and there is little use in applying it unless it can be continued for several months. Every effort is made to avoid reactions as evidenced by temperature rise, increased pulse-rate or other symptoms. Intolerance may also be shown by indefinite symptoms such as fatigue, loss of appetite, headache and malaise. The dosage is decreased or one or more injections omitted if symptoms of reaction appear. The treatment is without value in advanced tuberculosis. The results indicated are favorable in some cases, but the treatment is not a cure and should be employed as an addition to the other recognized methods of treatment.

Vaccination against Tuberculosis.—A great number of antigens have been tried. The general failure of all products obtained from the tubercle bacillus indicates that the bacillus, either live or dead, is the only type of vaccine which promises success. This would also be indicated by the facts presented above (see Immunization), that immunity depends on the sensitization which follows tubercle formation. In other words, can artificial inoculation be applied safely to induce tubercle formation and thus avoid the haphazard and frequently dangerous implantations which occur under natural conditions of exposure?

For this purpose live virulent, live attenuated and dead bacilli have been employed.

Webb and Williams (1908) used live virulent human-type bacilli, giving children minute doses at intervals. This procedure is not likely to be adopted because of the fear of the possibility of dissemination of the virulent bacilli.

Dixon (1889), Grancher and Ledoux-Lebard (1890) and Trudeau (1893) showed that the injection of attenuated bacilli would produce an immunity in animals. With the discovery of the different types of tubercle bacilli and the relative avirulence of the human type for cattle, there naturally developed an attempt to immunize cattle by injecting the human type. Unfortunately it was found that bacilli thus injected may be harbored for a long time and may pass to the udder (Smith and Griffith). The non-pathogenic acid-fasts from fishes, turtles, etc., have been tried, but these gave little or no immunity. Many methods of attenuating bacilli have been tried. At present attention is focussed on the Calmette-Guerin method of cultivation on glycerin-bile-potato medium.

The use of killed bacilli dates back to Koch. The demonstration by Prudden and Hodenpyl (1891) that dead bacilli give rise to tubercle formation, and by Babes and Proca (1896), that this may result in tuberculin sensitivity, indicates that the procedure is theoretically sound. Trudeau found that the resulting immunity is less pronounced than that following the injection of living attenuated bacilli. Several investigators have used killed bacillus vaccines in children. Raw injected 412 children and in the succeeding four years none died of tuberculosis. Skin sensitiveness develops. Petroff has recently made an intensive study of the sensitizing and immunizing effect of dead

tubercle bacilli. He favors this method of immunization on the ground that attenuation of virulence living bacilli may not be permanent, that is, that there is the possibility of reversion in the body.[1]

At present the use of the B. C. G. (Bacillus Calmette-Guerin) vaccine given by mouth, intradermally or subcutaneously, is attracting great attention.

Results of the Use of ihe Feeding the Calmette Vaccine to Infants.—The use of the vaccine in France and in countries in close sympathy with France has been very great. More than 100,000 infants have been fed the vaccines. Three feedings are given within the first ten days of life. If possible, the infants are removed from their tuberculous surroundings for a month to give the vaccine time to immunize. The European statistics are generally recognized as not being accurate. The mortality from tuberculosis among the vaccinated babies is reported about one-twentieth of that among the unvaccinated ones. The older children in whom more than three years have passed since they were vaccinated are in good condition, and there is not the slightest evidence of the tubercle bacilli remaining and becoming more virulent in their bodies, such as some observers like Petroff[2] fear.

In New York City Kereszturi, working in coöperation with us, has in the most careful manner followed up treated cases and control cases. One hundred and eighty babies have been fed the vaccine by the mouth. Only 2 of these have died from tuberculosis. In 1 an autopsy was obtained and the culture was shown to be of the human type. This baby was perfectly well up to the end of five months, when it developed whooping cough and later generalized tuberculosis. The results in the unvaccinated babies are not as good, nearly 10 per cent. of the babies dying from tuberculosis. We find, however, on carefully examining the statistics that a greater percentage of the control babies were in families in which open tuberculosis was present. At the present time our impression is that the vaccination is safe, but gives only a moderate degree of protection. About one-half of the babies develop after the third month a transient and moderate positive tuberculin reaction. We are beginning to vaccinate the older infants with small subcutaneous or intracutaneous injections, giving from $\frac{1}{100}$ to $\frac{1}{10}$ mg. We were encouraged to do this because of the tendency in Europe by some investigators to try this form of inoculation and because animals showed better results from the subcutaneous than from the feeding experiments.

The difficulty met with in determining the value of feeding and injecting the vaccine in children has caused a number of animal experiments.

Instead of reporting the earlier results of European observers, we give the following statement of our own results. We were able to produce small discrete non-progressive tubercles in guinea-pigs and monkeys which were vaccinated subcutaneously and intraperitoneally. In no

[1] For fuller survey and bibliography, see Petroff: Jour. Am. Med. Assn., 1927, **89**, 285.
[2] Petroff, S. A., and Branch, A.: Am. Jour. Pub. Health, 1928, **18**, 7, 843.

instance were these lesions reproduced upon guinea-pig subinoculation. Some other observers have had positive results from subinoculations. After subcutaneous injection of the vaccine we were able to demonstrate progressive tuberculosis in only one guinea-pig out of a large number. This animal had a subacute pneumonia which was the actual cause of its death. It probably reduced the resistance of the guinea-pig and made it more susceptible to the large dose of tubercle bacilli.

The results in the attempt to vaccinate animals subcutaneously were fairly successful. While vaccinated animals which were later infected with virulent organisms, as a rule, developed more or less progressive tuberculosis, they developed the disease in almost every instance to a much less extent than non-vaccinated controls which received the same amount of virulent organisms.

So far as the feeding results go in new-born guinea-pigs and calves, no evidence has been shown of infection with the vaccine and very little of protection. Two of 8 calves in this group did, however, show evidence of protection. In fairly young but not new-born monkeys, on the other hand, there was evidence of slight absorption; 4 monkeys out of 9 which were fed the vaccine were found at autopsy to have a few localized tubercles, usually in the mesenteric nodes. These findings indicate that the bacilli in the vaccine may pass the mucous membrane of the intestinal tract of monkeys. The fact that inoculation of these tubercles into normal guinea-pigs failed in every case to infect indicates the successful attenuation of our B. C. G. cultures when grown continuously on the bile medium of Calmette.

It is interesting to note that none of the guinea-pigs and calves fed the vaccine developed hypersensitiveness to tuberculin, while a large majority of those receiving the B. C. G. subcutaneously and intraperitoneally became allergic. The hypersensitiveness usually appeared by the eighth week and seemed to last on an average from six to ten months. In monkeys, in contrast to guinea-pigs and calves, the bacilli in the vaccine passed the intestinal mucosa, developed hypersensitiveness and produced localized tubercles in the mesenteric nodes. We feel certain that the positive findings in the orally vaccinated monkeys arose from the B. C. G., since 24 normal control monkeys when kept the same length of time failed to react to tuberculin and were negative at autopsy.[1]

Antituberculous Serum.—Every conceivable way of obtaining the true products of the tubercle bacilli has been tried, so as to cause the injected animals to produce antibodies both antitoxic and bactericidal. In spite of much conflicting testimony, it is probably safe to assert that no serums now obtainable have any great value.

Auto-urine Test.—The presumption that tubercle bacillus products are excreted in the urine has been the subject of experimental test. Windholz[2] using urine from tuberculous cases, concentrating it at a low temperature under reduced atmospheric pressure, induced skin reactions by intracutaneous injection of the patient from whom the urine was

[1] King and Park: Am. Jour. Pub. Health, 1929, **19**, 179.
[2] Corr.-Bl. f. schweiz Aertzte, 1919, **49**, 793.

obtained or of other patients. He believed that the presence of such reaction-inducing substances could be used as an indication of the presence of active tuberculous diseases. The reports by other observers[1] have been conflicting. Although many of the reactions obtained are specific, non-specific or pseudoreactions also occur.

Complement-fixation Reaction and Other Serum Tests for Diagnosis.—The results obtained with the agglutination reaction by various observers have been very conflicting. At present the test cannot be advised as useful in diagnosis, as the sera of cases suffering from tuberculosis frequently fail to give a reaction, while the sera from those having no detectable tuberculosis frequently cause a good reaction. A reaction in dilutions of 1 to 10 or 1 to 15 is considered a positive test. Concentrated extracts of tubercle bacilli gives a precipitation reaction in a fair proportion of cases. It has only a limited value as a diagnostic procedure. (For Complement Fixation see Chapter XIV.)

The Tubercle Bacillus of Domestic Animals and its Relation to Human Tuberculosis.—Among the domestic animals tuberculosis is most common in cattle. On account of the milk which they provide for our use, and which is likely to contain bacilli, the relation of these to human tuberculosis is a matter of extreme importance.

The chief seat of the lesions is apt to be the lymphatic nodes or lungs, and with them the pleura; less often the abdominal organs and the udder are affected. In pigs the abdominal organs are more often involved, then the lungs and lymphatic glands. In sheep, horses and goats tuberculosis is rare.

Differences between Tubercle Bacilli of Human and Bovine Type.—As has been already noted in the tables given of the incidence of bovine and human infection, it is possible to tell in any case the type of infection. The essential differences are in cultural characteristics and in virulence for rabbits and calves.

Cultural Differences.—The bovine bacillus grows very poorly when isolated, the human bacillus very freely. This is noted on plain egg, but to a less extent than on glycerin egg. The glycerin restrains or adds little to the growth of bovine bacilli, but increases markedly the amount of growth of the human bacillus. In fact, primary cultures on glycerin egg of bovine material commonly fail to grow. This difference is very noticeable in the first few generations and is sufficient in the great majority of instances for differentiation to one who has had some experience with such cultures. Further, the majority of human strains can be transplanted to glycerin potato or glycerin broth and give vigorous growth in the first few generations, whereas the bovine bacillus fails to grow or growth is very slight. After further cultivation the bovine bacillus gradually increases its amount of growth until it may be indistinguishable from the human type. This increase in luxuriance may be rapid or very slow.

[1] Gibson and Corroll: Jour. Am. Med. Assn., 1921, p. 1381. Lewis: Am. Jour. Med. Sci., 1923, **165**, 890.

Rabbit Virulence.—The bovine bacillus is exceedingly virulent for rabbits by any method of inoculation; the human bacillus only slightly so. The best method of differentiation is by intravenous inoculation. A small amount of culture is weighed after the moisture has been extracted with filter paper and a suspension made in normal saline and diluted so that 1 c.c. = 0.01 mg. of culture; this amount is then injected into the ear vein of a rabbit. If the rabbit survives for from forty to fifty days and on autopsy shows only lesions in the lungs or kidneys or both, the strain is of the human type. With the bovine type of bacillus the rabbit will die in the majority of instances before or about this time, if not it may be killed. On autopsy a progressive generalized tuberculosis will be found. The lesions in the lungs will be very marked, the tubercles having become confluent with caseous centers. The liver or spleen or both will be peppered with tubercles. Tubercles will be present in the great majority of cases in the superficial lymph nodes and also in those of abdomen and thorax. There may be tubercles on the heart, in the rib marrow or over the peritoneum.

These two differences alone are sufficient to differentiate in every case the type of bacillus. It must be insisted upon again that the cultural characteristics be observed in the early generations and, further, that the virulence be tested in early generations. In case the bovine culture does not afford sufficient material for weighing, a suspension can be made and compared with a weighed suspension.

Virulence for Calves.—In proving the non-identity of the two bacilli, calf experiments were resorted to. This was necessary as the supposed bovine cultures from children would have to be virulent for calves to the same extent as cultures from bovine material. The commonly used method was the subcutaneous inoculation in the side of the neck with 50 mg. of culture. The human type of bacillus caused only a local lesion or at most a spreading to the nearest lymph node. The bovine bacillus, on the other hand, caused a generalized tuberculosis which was or was not fatal. Sufficient data has been accumulated to make this test practically unnecessary for the determination of type.

Differences in Morphology.—The bovine bacillus tends to be shorter, thicker and solidly stained; the human type tends to be longer, slimmer, usually bent and shows beading and irregularities in staining. We have found this difference most marked on glycerin egg, slight or imperceptible on other media.

Besides the above differences Theobald Smith made the interesting discovery that the production of acid differed with the two types when grown on glycerin broth. The bovine type renders the bouillon less and less acid; this may even progress until the medium becomes slightly alkaline to phenolphthalein. The human type causes a preliminary fall in the acidity; as growth progresses the acidity is then gradually increased and may exceed the original acidity of the broth used. This difference is evident in tuberculin made from the two types of bacilli. The bovine tuberculin is alkaline or very slightly acid while human tuberculin is markedly acid. The change is only noticed when glycerin

is used in the media. The work of more recent investigators would seem to show that this difference, like all differences between the types, is quantitative and that intermediate reactions occur.

Bird (Avian) Tuberculosis.—Tuberculosis is very common among fowl. The bacillus grows easily and freely on glycerin media. It tends to form a moist or even slimy growth, and commonly produces an orange pigment. It is able to grow at a higher temperature than mammalian tubercle bacilli, the latter failing to grow above 41° C., the former growing at even higher temperatures. Guinea-pigs are less susceptible to inoculation with avian tubercle bacilli, and the virulence for these animals is usually quickly lost. Rabbits are much more susceptible. Rats and mice are spontaneously infected with avain tubercle bacilli and are supposed to be an important factor in spreading the disease. Birds are refractory, with few exceptions, to infection with the mammalian tubercle bacillus. Parrots, however, are susceptible to infection with all three types and commonly have spontaneous tuberculosis caused by the human type of bacillus.

Stability of the Different Types of Bacilli.—The fact that the agglutination reactions and the tuberculin reactions of the different types is similar shows their close relationship. This has led to the endeavor to change one type into the other. This is usually attempted by passage through animals. Some human-type cultures have been passed through a series of calves without any change except for a moderate increase in virulence. Other cultures seem to have changed their type completely. We believe that this is not a change of type, but an additional bovine infection. Strong negative evidence is the fact that the bovine bacillus when infecting man loses none of its characteristics, though present in the human body for years.

Tuberculosis of Cold-blooded Animals.[1]—The bacilli of this group are of interest mainly because of such claims as that of Friedmann, that immunity against the tubercle bacillus is produced by their injection. A similar attempt to employ the cold-blooded types for immunization of cattle was advocated by Klimmer.

These types have been isolated from spontaneous, tuberculous-like lesions of frogs, lizards, turtles, fishes, etc. They have little resemblance to the mammalian types of tubercle bacilli other than their acid-fastness. They grow rapidly and luxuriantly on ordinary media and their optimum of growth is at 20° to 30° C., higher temperatures inhibiting their growth. They are not pathogenic for warm-blooded animals, although toxic symptoms or limited lesions may be produced by the injection of large doses as with most other non-pathogenic acid-fasts. Tuberculins prepared from them are not toxic for tuberculous mammalia except in large doses, so that a specific reaction can be excluded. It is possible but very improbable that they might stimulate the production of some antibodies which would protect against mammalian bacilli to a limited extent.

Methods of Examination for Tubercle Bacilli.—One of the most important results of the discovery of the tubercle bacillus relates to the

[1] See large table for new names and characteristics, opposite p. 307.

practical diagnosis of tuberculosis. The staining peculiarities of this bacillus render it possible by the bacteriological examination of microscopic preparations to make an almost positive diagnosis in the majority of cases. A still more certain test in doubtful cases is the subcutaneous or intraperitoneal injection of guinea-pigs, which permits of the determination of the presence of numbers of bacilli so small as to escape detection by microscopic examination. For the animal test, however, time is required—at least three weeks, and, if the bacilli present are very few in number, at least six weeks—before any positive conclusion can be reached.

Microscopic Examination of Sputum for the Presence of Tubercle Bacilli.—1. **Collection of Material.**—The expectoration discharged in the morning is to be preferred, especially in recent cases, and the material collected should be coughed up from the lungs. Care should be taken that the contents of the stomach, nasopharyngeal mucus, etc., are not discharged during the act of expectoration and collected instead of pulmonary sputum. If the expectoration be scanty the entire amount discharged in twenty-four hours should be collected. In pulmonary tuberculosis the purulent or muco-purulent sputum usually contains bacilli; while pure mucus, blood and saliva, as a rule, do not. When hemorrhage has occurred, if possible some purulent or muco-purulent sputum should be selected for examination. Delay does not vitiate the results so far as the examination for tubercle bacilli is concerned. If, however, a determination of the associated bacteria is desired it is necessary to examine it in as fresh a condition as possible and the sputum particles should be washed to remove mouth contaminants.

2. **Methods of Examination.**—*Examination for Tubercle Bacilli.*—Pour the specimen into a clean, shallow vessel, having a blackened bottom—a Petri dish placed upon a sheet of dull black paper answers the purpose—and select from the sputum some of the true expectoration, containing, if possible, one of the small white or yellowish-white masses or "balls." From this make medium-thick cover-glass or slide "smears" in the usual way. In doubtful cases a number of these particles should be smeared. The material is thick and tenacious and difficult to spread evenly. It may be spread by one glass slide over the other and sliding them apart. (For Methods of Staining, see p. 77.)

Occasionally one is able to demonstrate the presence of tubercle bacilli with the Hermann stain where Ziehl's carbol-fuchsin gives negative results. At least two smears should be made and examined, if possible, before a negative report is given. Many of the incipient cases will require several examinations before bacilli are found. Some will remain consistently negative. It must be remembered that lesions may exist and that without ulceration the bacilli do not find their way into the sputum.

Methods for Concentrating the Bacilli.—A difficulty is the uneven distribution of bacilli in the sputum. Rickards has shown that mere shaking for ten minutes will increase the positive findings through distribution of the bacilli. More efficient methods are based on the

digestion of the sputum and concentration of the bacilli. Uhlenhuth advises the use of antiformin, a patented preparation consisting of a mixture of sodium hydroxide and sodium hypochlorite solution. The following method, used at the Boston City Health Department, is described by Castleman:[1] One part of sputum and 2 of antiformin are mixed in container in which sputum was received at laboratory. The mixture is shaken for ten minutes in a shaker, then transferred to a centrifuge tube and centrifuged for ten minutes. The supernatant fluid is decanted, the tube refilled with water and again centrifuged. After decanting the supernatant fluid the washed sediment is spread on a slide, dried and stained as usual.

In addition to its solvent action, antiformin kills most of the bacteria in sputum but destroys tubercle bacilli only after prolonged exposure; the sediment may therefore be used for cultural purposes or inoculation into guinea-pigs.

In place of sedimenting the bacilli, the dissolved sputum may be shaken up with a hydrocarbon. When the hydrocarbon separates out from the sputum the waxy tubercle bacilli adhere to it and are collected in a layer between the dissolved sputum and the hydrocarbon. If the hydrocarbon is heavy, chloroform, they are carried down, if light, ligroin, they are carried up.

Kinyoun has modified the original ligroin method as given below. We have had very satisfactory results with its use. As a routine method it saves time and gives a high percentage of positive results.

Bottles of about 15 c.c. capacity containing about 2 c.c. of a 1 per cent. solution of cresol are used for collection. The cresol is added to limit decomposition of the sputum if its transit to the laboratory is delayed. When received 1 c.c. of ligroin (specific gravity not less than 0.715 or more than 0.72) is added and the bottle filled with an alkaline solution of hypochlorite of lime. If the bottle is full, about one-third must be poured out to allow for the addition of the solution. The hypochlorite solution is prepared as follows: Three packages of chlorinated lime are weighed, and for each 90 grams, 65 grams of sodium carbonate are taken. The lime is mixed with 500 to 600 c.c. of water and the carbonate is dissolved in 1500 c.c. of water by boiling. The carbonate is then added to the lime and thoroughly mixed. After standing twelve to twenty-four hours the solution is filtered off. The amount of available chlorine is estimated and the solution diluted so that the chlorine is 0.56 per cent. Then 7.5 grams of caustic soda are added to each 100 c.c. of the filtrate. The solution should be kept cool and in the dark. Fresh lots should be prepared about every three months.

After the addition of the ligroin and the solution of chlorine the bottles of sputum are placed in a shaking machine and thoroughly shaken for five to ten minutes. The bottles can then be allowed to stand until the ligroin rises, which takes several hours, or this can be hastened, placing the bottles in a centrifuge, with special cups to accommodate the bottles, and run for about ten minutes at moderate speed.

When the ligroin rises to the top a soapy layer develops at the point of contact with the fluidified sputum and the tubercle bacilli are collected in this layer. The soapy layer is taken up with a platinum loop and smears made on glass slides fixed by heat and stained. Individual slides must be used and the slides

[1] Am. Jour. Pub. Health, July, 1924, **14**, 610.

must be stained separately or error will result, as the bacilli are not firmly fixed to the slides.

Petroff recommends an equal part of 4 per cent. sodium hydroxide for digestion, the mixture being kept at 38° C. for fifteen to thirty minutes, shaking frequently to insure a uniform mixture.

All the antiformin methods must be used with caution, as it is easy to see how error can creep in from contamination with other acid-fast bacilli.

Detection of Tubercle Bacilli in Urine and Feces, Etc.—The catheterized urine is centrifugalized. If little sediment appears, the upper portion of the fluid is removed and more urine added and again centrifugalized. If the urine is rich in salts of uric acid, the same may be diminished by carefully warming the urine before treating it. If too alkaline add a little acetic acid. A possible source of error is the presence of smegma bacilli.

The feces are examined for any purulent or mucous particles. If none are found, larger masses of feces are removed and then the rest diluted and centrifugalized. The antiformin methods are a great aid in the examination of feces. The examiner must remember that bacilli swallowed with sputum may appear in the feces. Similar acid-fasts in foods must be considered.

In examining cerebrospinal fluid for tubercle bacilli it must be remembered that the majority of the bacilli are entangled in the delicate clot that forms. Whenever possible the fluid after withdrawal should be allowed to stand until this filmy clot develops, which is then fished out and examined. If this is impossible the fluid should be centrifugalized and the sediment stained. This is also the case in other serous fluids, but in ascitic or pleuritic fluid they are usually very few in number.

Inoculation of Animals.—The inoculation of suspected material into guinea-pigs produces tuberculosis; even if the number of bacilli is very small. When no bacilli can be detected by microscopic examination this can be done for diagnostic purposes. The material should be injected subcutaneously as already described.

Cultivation.—This requires so much time that it is not generally used except in important investigations upon the nature of the tubercle bacilli. The special methods have already been given under Media.

The Use of the Tuberculin Tests in Cattle for the Detection of Tuberculosis.—"Inspection should be carried on while the herd is stabled. If it is necessary to stable animals under unusual conditions or among surroundings that make them uneasy and excited, the tuberculin test should be postponed until the cattle have become accustomed to the conditions they are subjected to, and then begin with a careful physical examination of each animal. This is essential, because in some severe cases of tuberculosis, on account of saturation with toxins, no reaction follows the injection of tuberculin, but experience has shown that these cases can be discovered by physical examination.

The following procedure has been used extensively and has given excellent results:

"(*a*) Take the temperature of each animal to be tested at least three times, at intervals of two hours, before tuberculin is injected.

"(*b*) Inject in the evening, preferably between the hours of six and nine, 0.4 c.c. of Koch's tuberculin, previously diluted to 5 c.c. with sterile water. The injection should be made with a carefully sterilized hypodermic syringe. The most convenient point for injection is back of the left scapula.

"(*c*) The temperature should be taken eight hours after the injection, and temperature measurements repeated at regular intervals of two hours until the twenty-fourth hour after injection.

"(*d*) When there is no elevation of temperature at this time the examination may be discontinued; but if the temperature shows an upward tendency, measurements must be continued until a distinct reaction is recognized or until the temperature begins to fall.

The usual procedure at present is to make an annual test, using the tuberculin, both subcutaneously and intracutaneously. If there are any reactions the latter test is repeated at the end of two months. Six months after the performance of this annual test a semiannual intracutaneous test is done. Many are now using the Mantoux test altogether. For some reason a few exceptional cows will react to one of these tests and not to the other. Therefore, we urge that a subcutaneous test be done annually.

Method of Examining Milk for Tubercle Bacilli.—Thirty cubic centimeters of milk are centrifuged at high speed and 10 c.c. of the lower milk and sediment collected. Four cubic centimeters of the cream is thinned with a little sterile water and injected into two guinea-pigs. The sediment is injected in amounts of 3 to 5 c.c. into other pigs. Larger amounts than this are apt to kill too many pigs from the associated bacteria. Subcutaneous injection is to be preferred. There are certain precautions that must be taken in drawing conclusions, as the different types of acid-fast "butter bacilli" may cause lesions, and their presence will be noted in smears made from these lesions. To avoid this source of error, two methods are resorted to. If cultures are made from the suspected lesions on glycerin agar, these bacilli usually develop in a few days, whereas tubercle bacilli do not. When one is ready to kill the pigs, 2 c.c. of old tuberculin should be injected into each pig late in the day. The following morning the tuberculous pigs will be dead or dying. Autopsies should be done on all to confirm the test. The milk should be as fresh as possible to prevent the growth of other bacteria.

CHAPTER XXXI.

OTHER ACID-FAST BACILLI: BACILLUS OF LEPROSY, BACILLUS OF RAT LEPROSY, BACILLUS OF JOHNE'S DISEASE IN CATTLE, AND THE GROUP OF NON-PATHOGENIC ACID-FAST BACILLI.

LEPROSY BACILLUS—B. (MYCOBACTERIUM) LEPRÆ.

The bacillus of leprosy was discovered by Hansen and Neisser (1879) in the leprous tubercles of persons afflicted with the disease. This discovery was confirmed by many subsequent observers.

Morphology (in Tissues).—Small, slender rods resembling the tubercle bacilli in form, but somewhat shorter and not so frequently curved. The rods have pointed ends, and in stained preparations unstained spaces, similar to those observed in the tubercle bacillus, are seen. They *stain* readily with the anilin colors and also by Gram's method. Although differing slightly from the tubercle bacillus in the ease with which they take up the ordinary anilin dyes, they behave like tubercle bacilli in retaining their color when subsequently treated with strong solutions of the mineral acids and alcohol. The difference in staining characteristics is too slight to be relied upon for diagnostic purposes (see Plate VI).

Bacilli Isolated from Leprous Lesions.—No acid-fast organism was grown from leprous lesions until Clegg reported, in 1908, that he had been able to cultivate an acid-fast bacillus by growing it in symbiosis with ameba and cholera. Since then Duval, Kedrowski, Twort and many others have reported the finding of various more or less acid-fast organisms in leprous lesions. These organisms may be grouped as follows:[1]

Bacilli of the Diphtheroid Type.—In serum media the colonies are yellowish white and develop best at 37° C., although a slight growth occurs at room temperature. When growth occurs on broth, the medium remains clear and a pellicle is produced. The morphology is variable. They are either solidly stained or irregularly stained like other types of diphtheroids. They are Gram-positive and may show some resistance to decolorization after staining with carbol-fuchsin, especially the metachromatic granules.

Pathogenicity, none or questionable.

Acid-fast Chromogenic Bacilli.—This type of bacillus is difficult to isolate but after isolation grows freely at both 37° and 20° C. on

[1] For a fuller discussion and bibliography of earlier investigations, see the excellent résumé of Wollbach and Honeij: Jour. Med. Research, 1914, **29**, 367.

most of the ordinary media. The growth is luxuriant, moist and a yellow to deep orange color develops. The individual bacilli vary in morphology from coccoid to filamentous bacilli, some showing metachromatic granules, others showing clear areas. They are acid-fast but less so than the tubercle bacillus.

Pathogenicity.—Lesions claimed to be similar to leprosy produced in Japanese dancing mice and in monkeys.

Acid-fast Non-chromogenic Bacilli.—These types are characterized by their feeble, slow growth on artificial media and growth only takes place at 37° C. and then only on special media.

Morphologically they vary from plump to long, slender bacilli, often beaded or bipolar in appearance.

Pathogenicity, none.

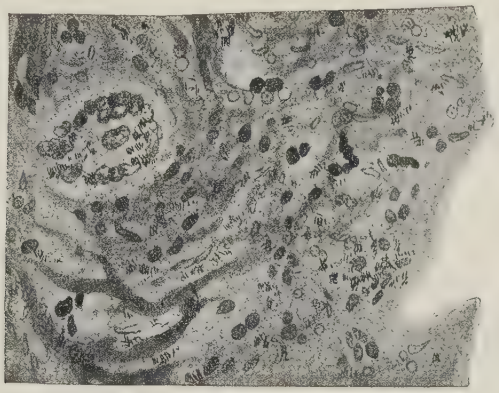


FIG. 151.—Leprosy bacilli in nodule. (Kolle and Wassermann.)

What conclusion is to be drawn from such variable results is difficult to say. Has the diphtheroid bacillus any relationship to the more acid-fast types? This is a possibility when one considers that under certain circumstances it shows some resistance to decolorization. Then, too, the leprosy bacilli in tissue may decolorize easily, although they are abundant, as shown by staining with polychrome methylene blue. On the other hand, the repeated isolation of diphtheroids from the lymph nodes in other conditions raises a strong element of doubt as to the etiological significance of this organism. A careful comparison of the diphtheroids from various conditions, especially their resistance to decolorization, might give us some help.

As to the chromogenic acid-fasts, the character of their growth, viz., the luxuriant growth upon ordinary media at low temperatures is not that of a highly specialized parasite. As to the apparently successful animal inoculations it must be remembered that lesions have been produced by acid-fast bacilli, known to be non-pathogenic. Why such bacilli should be frequently isolated from leprous lesions is still to be explained.

The non-pigmented types are more consistent with our idea of what the bacillus should be but whether they are actually the etiological organism remains to be seen.

The serum reactions, such as agglutination and complement fixation, have added no evidence as to the etiological significance of any one of the bacilli isolated. Each of the bacilli mentioned has been agglutinated by sera of lepers. The complement-fixation reactions await a successful specific test for the individual acid-fast organisms.

Pathogenesis.—Numerous inoculation experiments have been made on animals with portions of leprous tubercles, but there is no conclusive evidence that leprosy can be transmitted to the lower animals by inoculation. The inference that this bacillus bears an etiological relation to the disease with which it is associated is based chiefly upon the demonstration of its constant presence in leprous tissues.

The bacilli are found in all the diseased parts and usually in large numbers, especially in tubercles on the skin, in the conjunctiva and cornea, the mucous membranes of the mouth, gums, nose and larynx and in the interstitial processes of the nerves, testicles, spleen, liver and kidneys. The rods lie almost exclusively within the peculiar round or oval cells of the granulation tissues which compose the leprous tubercles, either irregularly scattered or arranged parallel to one another. In old centers of infection the leprosy cells containing the bacilli are larger and often polynuclear. Giant cells, such as are found in tuberculosis, are claimed to have been observed by a few investigators (Boinet and Borrel). In the interior of the skin tubercles, the hair follicles, sebaceous and sweat glands are often attacked and bacilli have sometimes been found in these (Unna, etc.). Quite young eruptions often show a few bacilli. A true caseation of the tubercles does not occur, but ulceration results. During acute exacerbations with development of new lesions bacilli have been observed in the blood.

In the anesthetic forms of leprosy the bacilli are found most commonly in the nerves and less frequently in the skin. They have been demonstrated in the sympathetic nervous system, in the spinal cord, and in the brain. The Bacillus lepræ occurs also in the blood, partly free and partly within the leukocytes, especially during the febrile stage which precedes the breaking out of fresh tubercles (Walters and Doutrelepont). The bacilli have also been found in the intestines, in the lungs and in the sputum, but not in the urine.

With regard to the question of the direct inheritance of the disease from the mother to the unborn child there is considerable difference of opinion. Some cases have been reported, however, in which a direct transmission of the bacillus during intra-uterine life seems to be the only or most plausible explanation of the infection. At the same time, we have no positive experimental evidence to prove that such an infection does take place. Although many attempts have been made to infect healthy individuals with material containing the bacilli of leprosy, the results are not conclusive. Even the experiments made by Arning, who apparently successfully infected a condemned criminal in the Sand-

wich Islands with fresh leprous tubercles, and which have been regarded as positive evidence of the transmissibility of the disease in this way, are by no means conclusive; for, according to Swift, the man had other opportunities for becoming infected. The negative results, together with the fact that infection does not more frequently occur in persons exposed to the disease, may possibly be explained by the assumption that the bacilli contained in the tuberculous tissues are mostly dead, or much more probably that an individual susceptibility to the disease is requisite for its production. None of the later attempts at cultivation have been more successful.[1]

The relation of leprosy to tuberculosis is sufficiently evident from their great similarity in many respects. This is rendered still more remarkable by the fact that leprosy reacts, both locally and generally, to an injection of tuberculin in the same manner as tuberculosis, but to a somewhat less extent.

Marchoux and Caro,[2] in reporting their positive results from a modification of Rubinson's (1926) diagnostic method of blood cell sedimentation, state that they obtained no results with tuberculous blood.

The latest reports on treatment with chaulmoogra oil and other measures are encouraging.[3, 4]

Rat Leprosy.—The interest in this disease lies in the fact that diphtheroid and chromogenic acid-fast bacilli similar to those described above have been isolated from leprous rats. Marchoux[5] reported leprous lesions in rat following injections of human leprous tissue. Henderson[6] reports a study of the pathology of rat leprosy, showing its similarity to the human disease.

Bacillus of Johne's Disease, Chronic Enteritis or Paratubercular Dysentery of Cattle.—This disease is comparatively common in this country and is characterized by chronic diarrhea and emaciation, commonly leading to death. The intestinal mucosa is thickened and the lesions are not limited. Tubercle formation and necrosis are absent. The bacilli are present in the lesions in enormous numbers. Twort succeeded in cultivating the organism and his work was verified by Holth and Meyer. The bacillus is not pathogenic for guinea-pigs or rabbits, although local abscesses may be produced.

Non-pathogenic Acid-fast Bacilli.—These have no importance further than historical interest and the fact that they may be present in materials suspected of containing tubercle bacilli and thus lead to error. They vary widely in their acid-fastness, especially when artificially cultivated. Differential staining methods have been devised to separate them from the tubercle bacillus and although in a general way the decolorization by prolonged action of acid and alcohol is presumptive evidence against suspected bacilli being tubercle bacilli, it is an unsafe procedure. Tubercle bacilli vary in their acid-fastness but the non-pathogenic types vary even more widely, some being extremely resistant to decolorization. Many of the non-pathogenic types grow rapidly at low temperatures and in cultures can thus be quickly differentiated from tubercle bacilli.

They can be separated from tubercle bacilli by inoculating animals in which no progressive lesions will develop, although limited lesions may be produced if

[1] Mello and Cabral: Bull. Soc. Path. Exp., 1926, **19**, 771.
[2] Ann. de l'Inst. Pasteur, 1928, **42**, 542.
[3] Lara: Jour. Philippine Island Med., 1928, **8**, 56.
[4] Rogers: Practitioner, 1928, **120**, 219.
[5] Bull. Acad. Med., 1925, **94**, 1091.
[6] Indian Jour. Med. Res., 1928, **16**, 1.

they are injected in large numbers. The guinea-pig may be injected with 2 c.c. of tuberculin and if infected with tuberculosis will die, but if with other acid-fast bacilli, will show little or no reaction. If a second group of guinea-pigs are inoculated with a small amount of the infected tissue from the inoculated pigs there will develop progressive tuberculosis if the doubtful bacilli were tubercle bacilli and practically no lesions if they were other acid-fast types. Cultures from the lesions may also be an aid in differentiation.

Bacillus of Lustgarten.—This bacillus was found by Lustgarten in 1884 in syphilitic lesions or ulcers. It is very similar morphologically to the smegma bacillus and may be identical with it.

Smegma Bacillus.—This bacillus is present in smegma from the prepuce or vulva. Its only interest is the danger of mistaking it for tubercle bacilli in the examination of urine, especially if the latter be carelessly collected.

Timothy and Other Grass Bacilli.—On various grasses, in cow manure, in butter and in milk there have been found bacilli with varying degrees of acid-fastness. Similar bacilli have also been demonstrated in water. They make the direct microscopic examination of such material for tubercle bacilli of little value and the nature of any acid-fast organisms so found must be determined by animal inoculations.

CHAPTER XXXII.

THE ANTHRAX BACILLUS

Anthrax is an acute infectious disease which is very prevalent among animals, particularly sheep and cattle. Geographically and zoölogically it is the most widespread of all infectious disorders. It is much more common in Europe and in Asia than in America. The ravages among herds of cattle in Russia and Siberia and among sheep in certain parts of France, Hungary, Germany, Persia and India are not equalled by any other animal plague. In this country the disease in the lower animals is rare.

The disease also occurs in man as the result of infection either through the skin, the intestines or in rare instances through the lungs. It is found in persons whose occupations bring them into contact with animals or animal products, as stablemen, shepherds, tanners, butchers and those who work in wool and hair. During the recent war the increased importation of skins, hides and bristles from foreign countries resulted in a much larger number of cases of anthrax. New shaving brushes have been frequently found abundantly infected with anthrax spores and many cases of infection from them have been reported. Efficient measures for the disinfection of suspected hides, bristles, etc., would largely prevent infections from this organism. Two forms of the disease have been described—the external anthrax, or malignant pustule, and the internal anthrax, of which there are intestinal and pulmonary forms, the latter being known as "woolsorters' disease."

Owing to the fact that anthrax was the first infectious disease which was shown to be caused by a specific microörganism and to the close study which it received in consequence, this disease has probably contributed more to our general knowledge of bacteriology than any other infectious malady.

Davaine, in 1863, announced to the French Academy of Sciences the results of his inoculation experiments and asserted the etiological relations of the microörganism to the disease. In 1877 Koch, Pasteur and others confirmed his statements by obtaining the bacillus in pure cultures and showing that the inoculation of these cultures produced anthrax in susceptible animals as certainly as did the blood of an animal recently dead from the disease.

Morphology.—Slender, cylindrical, non-motile rods, having a breadth of 1μ to 1.25μ and ranging from 2μ or 3μ to 20μ or 25μ in length. Sometimes short, isolated rods are seen and, again, shorter or longer chains or threads made up of several rods joined end to end. In suitable culture media very long, flexible filaments may be observed, which are frequently united in twisted or plaited cord-like bundles. (See

Figs. 152 and 153.) These filaments in hanging-drop cultures, before the development of spores, appear to be homogeneous or nearly so; but in stained preparations they are seen to be composed of a series of rectangular, deeply stained segments. When obtained directly from the blood of an infected animal the free ends of the rods are slightly rounded, but those coming in contact with one another are often quite square. In cultures the ends may be a trifle thicker than the body of the cell and somewhat concave, giving the appearance of joints of bamboo. At one time much stress was laid upon these peculiarities as distinguishing marks of the anthrax bacillus; but it has been found that they are the effects of artificial cultivation and not necessarily characteristic of the organism under all conditions. The bacillus is inclosed in a transparent envelope or capsule, which in stained preparations (from albuminous material) may be distinguished by its taking on a lighter stain than the deeply stained rods which it surrounds.

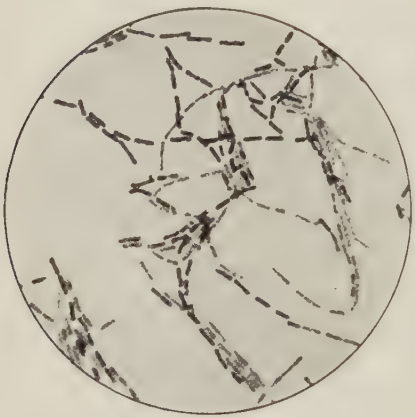


Fig. 152.—Anthrax bacillus. × 900 diameters. Agar culture.

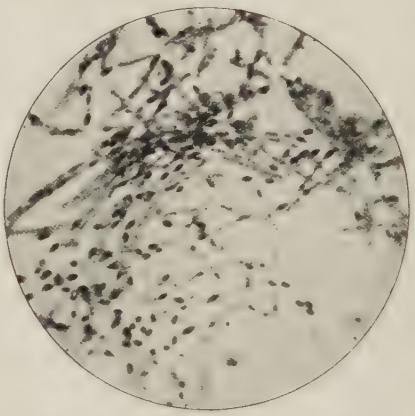


Fig. 153.—Spores heavily stained (in specimen red). Bodies of disintegrating bacilli faintly stained (in specimen blue). × 1000 diameters.

Motility.—The bacillus is non-motile. This serves to differentiate the anthrax bacillus from other biologically similar but non-pathogenic bacteria.

Spore Formation.—In the presence of free oxygen in cultures spores are developed in the bacilli. These spores are elliptic in shape and about one and a half times longer than broad. They first appear as small, refractive granules distributed at regular intervals, one in each rod. As the spore develops the mother-cell becomes less and less distinct, until it disappears altogether, the complete oval spore being set free by its dissolution. (See Fig. 153 and Plate II, Fig. 22.) Irregular sporulation sometimes takes place and occasionally there is no spore formation as in varieties of non-sporebearing anthrax.

Sporeless varieties have been produced artificially by cultivating the

typical anthrax bacillus under certain conditions, among which may be mentioned the addition of antiseptics, as carbolic acid and of continued high temperature (43° C.). Varieties differing in their pathogenic power may also be produced artificially. Pasteur produced an "attenuated virus" by keeping his cultures for a considerable time before replanting them upon fresh soil.

Anthrax cultures containing spores retain their vitality for years; in the absence of spores the vitality is much more rapidly lost. When grown in liquids rich in albumin the bacilli attain a considerable degree of resistance; thus dried anthrax blood has been found to retain its virulence for sixty days, while dried bouillon cultures only did so for twenty-one days. Dried anthrax spores may be preserved for many years without losing their vitality or virulence. They also resist a comparatively high temperature. Exposed in dry air they require a temperature of 140° C. maintained for three hours to destroy them; but suspended in a liquid most are destroyed in five to ten minutes by a temperature of 100° C. Some spores may resist for a longer period of time. Exposure to steam under pressure (15 pounds) for not less than ten minutes[1] is the preferable method of sterilizing spore-contaminated materials.

Staining.—The anthrax bacillus *stains* readily with all the anilin colors and also by Gram's method, when not left too long in the decolorizing solution. In sections good results may be obtained by the employment of Gram's solution in combination with carmine, but when only a few bacilli are present this method is not always reliable, as some of the bacilli are generally decolorized.

Biology.—The anthrax bacillus grows easily in a variety of nutrient media at a temperature from 18° to 43° C., 37° C., being the most favorable temperature. Under 12° C. no development takes place as a rule. Under 14° C. and above 43° C. spore formation ceases. The lower limit of growth and of sporulation is of practical significance in determining the question whether development can occur in the bodies of animals dead from anthrax when buried at certain depths in the earth. Kitasato has shown that at a depth 1.5 meters the earth in July has a temperature of 15° C. at most, and that under these conditions a scanty sporulation of anthrax bacilli is possible, but that at a depth of 2 meters sporulation no longer occurs. The anthrax bacillus is aërobic -that is, its growth is considerably enhanced by the presence of oxygen—but it grows also under anaërobic conditions, as is shown by its growth at the bottom of the line of puncture in stick cultures in solid media; but under these conditions it no longer produces the peptonizing ferment which it does with free access of air. Furthermore, the presence of free oxygen is absolutely necessary for the formation of spores, while carbonic acid gas retards sporulation. This explains, perhaps, why sporulation does not take place within the animal body either before or after death.

[1] Actual exposure.

Growth in Gelatin.—In *gelatin-plate cultures*, at the end of twenty-four to thirty-six hours at 24° C., small, white, opaque colonies are developed, which, under a low-power lens, are seen to be dark gray in the center and surrounded by a grayish, irregular border, made up of wavy filaments. As the colony develops on the surface of the gelatin these wavy filaments spread out, until finally the entire colony consists of a light gray, tangled mass, which has been likened to a Medusa head (Fig. 154).

At the same time the gelatin begins to liquefy and the colony is slowly surrounded by the liquefied medium, upon the surface of which it floats as an irregular, white pellicle. In *gelatin-stick cultures* at first, development occurs along the line of puncture as a delicate white thread, from which irregular, hair-like projections soon extend perpendicularly into the culture medium, the growth being most luxuriant near the surface, but continuing also below. At the end of two or three days liquefaction of the medium commences at the surface and gradually progresses downward.

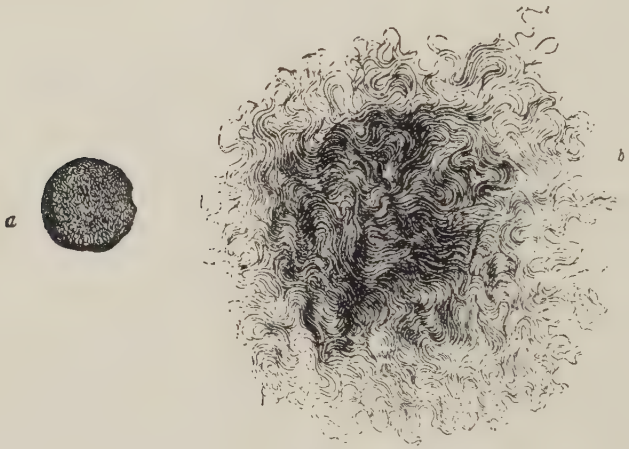


Fig 154.—Colonies of Bacillus anthracis upon gelatin plates: *a*, at the end of twenty-four hours; *b*, at the end of forty-eight hours. × 80. (F. Flügge.)

Growth on Agar.—The growth of *agar-plate cultures* in the incubator at 37° C. is similar to that on gelatin, and is still more characteristic and beautiful in appearance. A grayish-white layer is formed on the surface within twenty-four hours, which spreads rapidly and is seen to be made up of interlaced threads. Under the microscope the deep colonies are coarsely granular, dark brown, irregular in outline and show filamentous extension. The deep colonies are apt to be more distinctive when mixed cultures are being examined for B. anthracis.

Growth in Milk.—A small amount of acidity is produced. The milk is curdled and then digested by a rennin-like ferment.

Growth in Bouillon.—The growth is characterized by the formation of flaky masses, which sink as a sediment to the bottom of the tube, leaving the supernatant liquid clear.

Pathogenesis.—The anthrax bacillus is pathogenic for cattle, sheep (except the Algerian race), horses, swine, mice, guinea-pigs and rabbits. Rats,. cats, dogs, chickens, owls, pigeons and frogs are but little susceptible to infection. Small birds—the sparrow particularly—are somewhat susceptible. Man, though subject to local infection and occasionally to internal forms of the disease, is not as susceptible as some of the lower animals.

In the more susceptible animals the anthrax bacillus produces a true septicemia. Among test animals mice are the most susceptible, succumbing to very minute injections of a slightly virulent virus; next guinea-pigs and then rabbits, both of these animals dying after inoculation with virulent bacilli. Infection is most promptly produced by introduction of the bacilli into the circulation or the tissues, but inoculation by contact with wounds on the skin also causes infection. It is difficult to produce infection by the ingestion even of spores; but it may readily be caused by inhalation, particularly of spores.

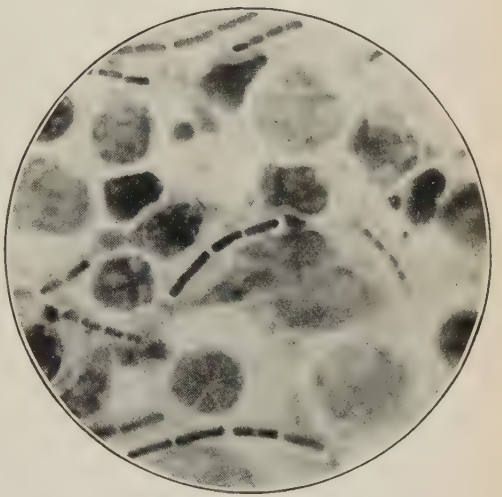


Fig. 155.—Spread from the liver of a mouse dead of anthrax septicemia. × 1700. Stained by Williams' rabies stain. Shows capsule. (Gerber and Siegel.)

Subcutaneous injections of these susceptible animals result in death in from one to three days. Comparatively little local reaction occurs immediately at the point of inoculation, but beyond this there is an extensive edema of the tissues. Very few bacilli are found in the blood in the larger vessels, but in the internal organs, and especially in the capillaries of the liver, the kidneys and the lungs, they are present in great numbers. In some places, as in the glomeruli of the kidneys, the capillaries will be seen to be stuffed full of bacilli and hemorrhages, probably due to rupture of capillaries by the mechanical pressure of the bacilli which are developing within them, may occur. The pathological lesions in animals infected by anthrax are not marked except in the spleen, which, as in other forms of septicemia, is greatly enlarged.

Occurrence in Cattle and Sheep.—Cattle and sheep are affected chiefly with the intestinal form of anthrax, infection in these animals commonly resulting from the ingestion of food containing spores.

The bacillus itself, in the absence of spores, is quickly destroyed by the gastric juice. The disease usually takes a rapid course and the mortality is high—70 to 80 per cent. The blood is very dark and tar-like. Bacilli are present, especially in the lymph spaces, in enormous masses.

Sheep are also subject to external anthrax, infection taking place by way of the skin; cattle are seldom infected in this way. At the point of inoculation there develops a hard, circumscribed boil—the so-called anthrax carbuncle; or there may be diffuse edema with great swelling of the parts.

Occurrence in Man.—The disease results from infection, either through the skin, the intestines or occasionally by inhalation through the lungs. It is usually produced by cutaneous infection through inoculation of exposed surfaces—the hands, arms or face. Infection of the face or neck would seem to be the most dangerous, the mortality in such cases being 26 per cent., while infection of the extremities is rarely fatal.

External anthrax in man is similar to this form of the disease in animals. There are two forms: malignant pustule or carbuncle and, less commonly, malignant anthrax edema.

In malignant pustule, at the site of inoculations, a small papule develops, which becomes vesicular. Inflammatory induration extends around this, and within thirty-six hours there is a dark, brownish eschar in the centre, at a little distance from which there may be a series of small vesicles. The brawny induration may be extreme. There may also be considerable edema of the parts. In the mildest form there may be only slight swelling.

Malignant anthrax edema occurs in the eyelids and also in the head and neck, sometimes the hand and arm. It is characterized by the absence of the papule and vesicle forms and by the most extensive edema.

The bacilli are found on microscopic examination of the fluid from the pustule shortly after infection; later the typical anthrax bacilli are often replaced by involution forms. In this case resort may be had to cultures, animal inoculation or examination of sections of the extirpated tumor. The bacilli are not present in the blood in large numbers until just before death.

Internal anthrax is much less common in man; it does, however, occur now and then. There are two forms of this: the intestinal form, or mycosis intestinalis and the pulmonic form, or wool-sorters' disease.

Intestinal anthrax is caused by infection through the stomach and intestines. The recorded cases of intestinal anthrax in man have occurred in persons who were in the habit of handling hides, hair, etc., which were contaminated with spores; in those who were conducting laboratory experiments and rarely it has been produced by the ingestion of food, such as raw ham and milk. The symptoms produced in this disease are those of intense poisoning, chill, followed by vomiting, diarrhea, moderate fever and pains in the legs and back. The pathological lesions are similar to those described in animals.

Wool-sorters' disease, or pulmonic anthrax, is found in large establishments in which wool and hair are sorted and cleansed, and caused by the inhalation of dust contaminated with anthrax spores. The attack comes on with chills, prostration, then fever. The pathological changes produced are swelling of the glands of the neck, the formation of foci of necrosis in the air passages, edema of the lung, pleurisy, bronchitis, enlargement of the spleen and parenchymatous degenerations.

Immunity against Anthrax Infection.—The question of the cause of the natural immunity in the dog and some other animals has been much studied. Phagocytosis is supposed to be the chief agent in these cases. In susceptible animals immunity may be produced artificially. The efforts of Pasteur to effect immunity in animals by preventive inoculations of a vaccine made of "attenuated virus" of the anthrax bacillus opened a new field of productive original research. Following in his wake, many others have devised methods of immunization against anthrax infection; but the one adopted by Pasteur, Chamberland and Roux has alone been practically employed on a large scale.

Anti-anthrax Serum.—Eichorn[1] has had the greatest experience in this country in preparing anti-anthrax serum in the horse. He uses the spore vaccine. The cultures used for the hyperimmunization of the horses are attenuated at a temperature of 42.5° C. for varying periods. From time to time they were tested for their pathogenicity by inoculation into mice, guinea-pigs and rabbits. The cultures which are removed from the incubator after twenty days of attenuation generally prove satisfactory, inasmuch as the test inoculation usually demonstrated their virulence for the mice and guinea-pigs, but not for rabbits.

The immunization of the horses is begun by the use of one or two doses of anthrax vaccine followed by the injection of very minute doses of virulent anthrax strains isolated from outbreaks of anthrax in animals throughout the United States and foreign countries. The injections are at first made subcutaneously and reactions carefully observed, the increase of dosage being regulated according to the reactions from previous doses. When considerable immunity has been developed the injections are then given intravenously and gradually increased in size until the animal is receiving 50 c.c. of a 50,000,000,000 suspension of the most virulent cultures of anthrax obtainable directly in the vein. This degree of immunity is obtained in from four to six months, depending upon the individual animal being immunized. When such time has elapsed the individual animals are test bled and the protective value of the serum established by the potency test of Ascoli upon guinea-pigs and rabbits. If a fair percentage of the guinea-pigs and rabbits are protected against ten lethal doses of virulent anthrax culture the animal is then bled, after which it is given regular cycles of injections and bleedings, the potency test being made at varying intervals. Following bleedings, two injections are given with an interval of one day, and ten days following the last injection of the next bleeding is done.

[1] Eichorn, A., Berg, W., and Kelser, R.: Jour. Agricul. Res., 1917, **8**, 37. Eichorn, A.: Jour. Am. Vet. Med. Assn., 1916, **48**, 669.

For the purpose of producing a spore vaccine it is desirable to use a peptone-free agar medium and after inoculation with the attenuated culture to grow the organism at a temperature of 37.5° C. for four to seven days, by which time an abundance of spores will have formed. The growth is then washed from the slants and collected in a sterile flask and heated at a temperature of 60° C. for one-half hour to destroy the vegetative forms of the organism. A measured quantity of this suspension can then be plated out in the usual manner and the spore content of 1 c.c. of the suspension established. A dilution can then be made to the desired amount for inoculation purposes. Thus, if it is desired to use for vaccination 1,000,000 spores, it is best to dilute the vaccine to a quantity of which 1 c.c. would contain this number. Of such vaccine 1 c.c. would constitute the dose for cattle and horses, with correspondingly smaller doses for calves and sheep.

In the preparation of spore vaccines it is essential to submit every lot to a test for pathogenicity by inoculating approximately 250,000 spores, that is, 0.25 c.c. of the standard suspension, into guinea-pigs and rabbits before employing the same for vaccination purposes. The guinea-pigs should die in from two to five days, whereas the rabbits should remain alive.

In consideration of the keeping qualities of the spore vaccine large lots can be prepared without fear of deterioration. In the bottling and storing of the same, however, proper care should be taken to prevent contamination.

The potency test is as follows: 5 guinea-pigs receive from 1 to 3 c.c. of serum subcutaneously and twenty-four hours later these 5 plus 2 check pigs receive about ten times the lethal dose of virulent anthrax broth culture. It is required that at least 3 of these pigs which received the serum should live and the 2 checks should die.

Serum Treatment.—Probably the largest amount of experience in this country has been by Symmers[1] at Bellevue Hospital. His practise is to consider every case as a possible case of septicemia until the culture results are obtained. He begins, therefore, by giving 150 c.c. of serum intravenously. The usual precautions are taken of warming the serum a little above the temperature of the body and giving the first few cubic centimeters very slowly. Until the blood culture results are obtained 30 c.c. of serum are given intramuscularly every four hours. If at the end of twenty-four hours the cultures are negative the serum injections are discontinued; if positive, 50 c.c. are continued for every four hours until cultures are negative. With this treatment the majority of cases of anthrax that come for treatment fairly early recover even after septicemia is present. The most recent report is the one by Regan,[2] of the effect of treating 6 cases with anti-anthrax serum.

Bacterial Cultures for Diagnosis.—The detection of the anthrax bacillus is ordinarily not difficult, as this organism presents morphological, biological and pathogenic characteristics which distinguish it from all

[1] Ann. Surg., 1922, **75**, 663.
[2] Am. Jour. Med. Sci., 1924, **167**, 266.

other bacteria. In the later stages of recovery from the disease, however, the bacilli may be absent or difficult to find and cultivation on artificial media and experimental inoculation in animals are not always followed by positive results. Even in sections taken from the extirpated pustule it is sometimes difficult to detect the bacilli. In such cases only a probable diagnosis of anthrax can be made. It should be remembered that the bacilli are not often found in the blood in large numbers until shortly before death and then only in varying quantity; thus blood examinations often give negative results, though the bacilli may be present in large numbers in the spleen, kidneys and other organs of the body. The suspected material should be streaked over nutrient agar plates and inoculated into melted agar for pour plates. It should also be inoculated in mice and rabbits. Direct smears should also be made. Such freshly opened lesions contain no spores so the material cannot be heated to get rid of contaminants.

Isolation from Hair or Bristles.—Here we may take advantage of the fact that the spores are present. The suspected material is rubbed up with salt solution in a mortar. Half of the emulsion is subjected to heat to kill vegetative contaminants. Both portions are then centrifuged and from the precipitates a number of dilutions in pour-agar plates are made, from which typical colonies are fished in from twenty-four to forty-eight hours and tested culturally and by animal inoculations. Special attention should be given to the deep colonies. (See Growth on Agar.) Mice and rabbits are also inoculated from the precipitates.

Differentiation From Other Similar Bacteria.—Among other bacteria which may possibly be mistaken for anthrax bacilli are *Bacillus subtilis* and the bacillus of malignant edema. The former is distinguished by its motility, by various cultural peculiarities and by being non-pathogenic. (See also description in chapter on Water Analysis.) The latter differs from the anthrax bacillus in form and motility, in being decolorized by Gram's solution, in being a strict anaërobe and in various pathogenic properties.

The diagnosis of internal anthrax in man is by no means easy, unless the history points definitely to infection in the occupation of the individual. In cases of doubt, cultures should be made and inoculations performed in animals. Rabbits are more specifically susceptible than mice.

Precipitin Reaction.—Ascoli,[1] taking advantage of the known fact that bacterial precipitinogen is resistant to heat developed a diagnostic procedure of considerable value. An immune anthrax serum is employed of sufficient titre to give rapid precipitation but it should not have an unusual content of common antibodies, or confusing reactions will be obtained with other than the anthrax bacillus. That is, the reaction is not completely but only quantitatively specific. The suspicious lesion is broken up and boiled with 5 to 10 parts of saline, the extract filtered through paper or cleared by centrifuge. In place of salt solution, a dilution of 1 to 1000 acetic acid may be employed. In making extracts

[1] Zeitsch. f. Immunforsch, 1911, **11**, 103.

from agar cultures we have found that suspending and extracting the growths in 5 c.c. of salt solution gives satisfactory results. The extract is superimposed on the serum to elicit a ring reaction. With experience and knowledge of the degree of specificity of the serum employed very reliable results are obtainable. The method is of especial value in *inspection control of meat.* Old or decomposed lesions will still give a reaction. Naturally a negative result with tissue extracts has not the same value as a positive reaction. The number of bacilli and the amount of bacillary products in the tissue must be considerable before a definitely positive reaction can be obtained.

CHAPTER XXXIII.

ANAËROBIC BACILLI.

(Genus Clostridium.)

In this chapter we are dealing with the large spore-bearing anaërobic bacilli which have been grouped as the genus Clostridium. Those of most interest to us are B. (Clostridium) tetani, C. botulinum, and the gas gangrene group of bacilli.

In 1918 von Hibler divided these anaërobes into two not well-defined groups, the proteolytic and the saccharolytic. Members of the former as a rule liquefy coagulated blood serum, produce varying amounts of H_2S and digest milk. The saccharolytic bacilli do not digest coagulated serum or milk but they ferment on the whole carbohydrates more energetically than the other group, with the production of large amounts of gas. In the proteolytic group are included B. tetani, the botulinus group, B. putrificus, B. sporogenes and B. histolyticus. The saccharolytic group includes the most pathogenic of the anaërobes found in war wounds, namely, Vib. septique, B. welchii, B. œdematiens and B. fallax. It also includes B. chauvei.

THE BACILLUS AND THE BACTERIOLOGY OF TETANUS.

Tetanus is a disease which is characterized by a gradual onset of general spasms of the voluntary muscles, commencing in both man and the horse most often in the muscles of the jaw and neck and extending in severe cases to the muscles of the body. The disease is usually associated with a wound received from four to fourteen days previously.

In 1884 Nicolaier, under Flügge's direction, produced tetanus in mice and rabbits by the subcutaneous inoculation of particles of garden earth. The Italians, Carle and Rattone, had just before demonstrated that the pus of an infected wound from a person attacked with tetanus could produce the same disease in rabbits. Finally, Kitasato, in 1889, obtained the bacillus of tetanus in pure culture and described his method of obtaining it and its biological characters.

Occurrence.—The tetanus bacillus occurs widely throughout the world as a common inhabitant of the soil, especially in places where manure has been thrown. It has been found in many different substances and places—in hay dust, in horse and cow manure (its normal habitat is the intestine of the herbivora), in the mortar of old masonry, in the dust from horses' hair, in the dust in rooms of houses, barracks and hospitals, in courtplaster (McCoy[1]), occasionally in gelatin and in catgut.

[1] Pub. Health Rep., U. S. Pub. Health Service, 1917, **424**, 1450.

The tetanus bacilli are more numerous in certain localities than in others—for example, some parts of Long Island and New Jersey have become notorious for the number of cases of tetanus caused by small wounds—and they are fairly common in New York City. As a rule they are more abundant in regions where the temperature is high. In some islands and countries in the tropics cases of puerperal tetanus and tetanus in the newborn are very frequent. Tetanus bacilli are found in the intestines of about 15 per cent. of horses and calves living in the vicinity of New York City. They are also present to a somewhat less extent in the intestines of other animals and of man. Noble[1] gives a good review of their incidence.

Morphology.—From young cultures the bacilli appear as moderately slender rods, with rounded ends, 0.3μ to 0.5μ in diameter by 2μ to 4μ in length, usually occurring singly, but, especially in old cultures, often growing in long threads. They form round or nearly round spores, thicker than the cell (from 1μ to 1.5μ in diameter), occupying one of its extremities and giving to the rods the appearance of small pins (Fig. 156.)

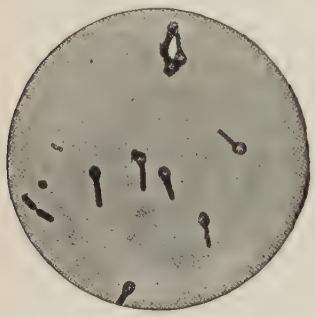


Fig. 156.—Tetanus bacilli with spores distending ends. × 1100 diameters.

Staining.—The bacillus is *stained* with the ordinary anilin dyes, and is moderately Gram-positive. The spores are readily stained by any spore stain (see Chapter on Stains). The flagella are fairly easily stained on freshly developed bacilli obtained by frequent transplants at short intervals.

Biology.—An *anaërobic, liquefying, moderately motile* bacillus. It has abundant peritrichic flagella. During the period of spore formation it is not motile. It grows slowly at temperatures from 20° to 24° C. and best at 38° C., when, within twenty-four to thirty hours it forms spores. At temperature of 20° to 24° C., spores form in from six to ten days. It grows well in an atmosphere of hydrogen gas. If planted with certain other bacteria that use up free oxygen the tetanus bacillus grows luxuriantly without a seal in deep tubes.

Growth in Media.—The bacillus of tetanus grows in nutrient media of a slightly alkaline reaction. The addition to the media of 1.5 per cent. of glucose causes the development to be more rapid and abundant. Chopped meat medium (see Chapter on Media) is very favorable for growth. On *gelatin plates* the colonies develop slowly; they resemble somewhat the colonies of the *Bacillus subtilis*. They have a dense, opaque center surrounded by fine, diverging rays. Liquefaction takes place more slowly, however, than with Bacillus subtilis and the resemblance to these colonies is soon lost.

The colonies on *agar* are quite characteristic. To the naked eye they present the appearance of light, fleecy clouds; under the microscope, a tangle of fine threads.

The *stab cultures in gelatin* exhibit the appearance of a cloudy, linear mass,

[1] Jour. Inf. Dis., 1915, **16**, 132.

with prolongations radiating into the gelatin from all sides (arborescent growth) Liquefaction takes place slowly. In *stab cultures in agar* a growth occurs presenting the appearance of a miniature pine tree. *Alkaline bouillon* is rendered somewhat turbid by the growth of the tetanus bacillus. In all cases, whether sugar is present or not, a production of gas results, accompanied by a characteristic and very disagreeable odor. It develops in *milk* without causing coagulation. Brain medium is slightly blackened denoting the presence of H_2S.

Resistance of Spores to Deleterious Influences.—The spores of the tetanus bacillus are very resistant to outside influences; in a desiccated condition they may retain their vitality for several years and are not destroyed in two and a half months when present in putrefying material. The spores show a variable but high resistance to heat. As a rule, one hour exposure to moist heat will kill. Occasionally spores may survive boiling for seventy minutes. All spores are killed by exposure to dry heat, 160° C. for one hour, or to steam under pressure, 120° C., for twenty minutes. (See also table in chapter on Disinfection.)

With regard to the persistence of tetanus spores upon objects where they have found a resting place, Henrijean reports that by means of a splinter of wood which had once caused tetanus he was able after eleven years again to cause the disease by inoculating an animal by means of the same splinter.

Pathogenesis.—In mice, guinea-pigs, rabbits, horses, cattle, goats and a number of other animals, inoculations of pure cultures of the tetanus bacillus cause tetanus after an incubation of from one to four days. In the smaller animals tetanus usually develops first in the muscles nearest the point of inoculation. A mere trace of an old culture—only as much as remains clinging to a platinum needle—is often sufficient to kill very susceptible animals like mice and guinea-pigs. Other animals require a larger amount. Rats and birds are but little susceptible and fowls scarcely at all. These never develop tetanus from natural infection. It is a remarkable fact that an amount of toxin sufficient to kill a hen would suffice to kill 500 horses. It is estimated that if 1 gram of horse requires 1 part of toxin to kill, then 1 gram of guinea-pig requires 6 parts, 1 of mouse 12, of goat 24, of dog 500, of rabbit 1500, of cat 6000, of hen 360,000. Horses frequently develop tetanus after injuries or operations. Cultures from different cases vary in their toxicity. On the inoculation of less than a fatal dose in test animals a local tetanus may be produced, which lasts for days and weeks and then ends in recovery. On killing the animal there is found at autopsy, just at the point of inoculation, a hemorrhagic spot, and no changes, other than this. A few tetanus bacilli may be detected locally with great difficulty, often none at all; possibly a few may be found in the region of the neighboring lymphatic glands and even in the blood. From this scanty occurrence of bacilli the conclusion has been reached that the bacilli of tetanus, when inoculated in pure culture, do not multiply to any great extent in the living body, but only produce lesions through the absorption of the poison which they develop at the point of infection. It has been found that pure cultures of tetanus, after the germs have sporulated and the toxins been destroyed by heat, can be injected into animals without producing tetanus. But if pathogenic streptococci or staphylo-

cocci or even non-pathogenic organisms are injected simultaneously with the spores, or if there is an effusion of blood at the point of injection, or if there was a previous or later bruising of the tissues, the animals surely die of tetanus.

Natural Infection.—Here the infection may be considered as probably produced by the bacilli in their spore state and the conditions favoring infection are almost always present. A wound of some kind has occurred, penetrating at least through the skin, though perhaps of a most trivial character, such as might be caused by a dirty splinter of wood, and the bacilli or their spores are thus introduced from the soil in which they are so widely distributed. If in any given case, the tissues being healthy, the ordinary saprophytic germs are killed by proper disinfection at once, a mixed infection does not take place, and tetanus will not develop. If, however, other bacteria, especially pathogenic or putrefactive types accompany the tetanus bacilli, or if the tissues infected be bruised or lacerated, the spores may develop and produce the disease.

Tetanus in Man.—Man and almost all domestic animals are subject to tetanus. It is a comparatively rare disease in the United States, during times of peace, except after the Fourth of July celebration, when a considerable number of cases develop. Injuries causing crushing of tissues with accompanying contamination are the most likely to be followed by tetanus. Until immunization became the practice more than one hundred persons yearly developed tetanus after blank cartridge wounds. On examination of an infected individual very little local evidence of the disease can be discovered. Generally at the point of infection, if there is an external wound, some pus is to be seen, in which, along with numerous other bacteria, tetanus bacilli or their spores may be found. Although rather deep wounds are usually the seat of infection, at times such superficial wounds as an acne pustule or a vaccination may give the occasion for infection. In rare cases tetanus has developed from the infection of necrotic mucous membranes as in diphtheria. Not only undoubted traumatic tetanus, but also all the other forms of tetanus are now conceded to be produced by the tetanus bacillus—puerperal tetanus, tetanus neonatorum and idiopathic tetanus. In tetanus neonatorum, infection is introduced through the navel, in puerperal tetanus through the inner surface of the uterus. It should be borne in mind that when there is no external and visible wound there may be an internal one. The lesions in the nervous system are still obscure. Congestion, cellular exudate into the perivascular spaces and chromatolysis of the ganglion cells are common.

Toxins of the Tetanus Bacillus.—It is evident from the localization of the tetanus bacilli almost wholly at the point of inoculation and their moderate multiplication at this point that they exert their action through the production of a powerful toxin. In fact the bacilli produce at least two toxins named, according to their action, the tetanospasmin and the tetanolysin. But the first only is of importance. Tetanolysin merely produces some lysis of the red blood cells of various animals. An antihemolysin has been produced.

Tetanospasmin is an extremely powerful exotoxin. One ten-thousandth of a cubic centimeter of the filtrate of an eight-day bouillon culture of a fully virulent bacillus is sufficient to kill a mouse. The purified and dried tetanus toxin prepared by Brieger and Cohn was surely fatal to a 15-gram mouse in a dose of 0.000005 gram. The toxin is precipitated by saturating the broth with ammonium sulphate and having been collected on the filter is compressed to eliminate the fluid clinging to it. The appalling strength of tetanus toxin may readily be appreciated when it is stated that it is twenty times as poisonous as dried cobra venom.

The quantity of the toxin produced in nutrient media varies according to the age of the culture, the composition of the culture fluid, reaction, completeness of the exclusion of oxygen, etc. For some reason more toxin develops in broth inoculated with masses of tetanus spores in nutrient agar, than with bacilli. The variation in strength is partly due to the extreme sensitiveness of the toxin, which deteriorates on keeping at blood heat or on exposure to light. It is sensibly affected by most chemical reagents and is largely destroyed by heating to 55° to 60° C. for a few minutes. It retains its strength best when protected from heat, light, oxygen and moisture.

The tetanus cultures retain their ability to produce toxin unaltered when kept under suitable conditions; but when subjected to deleterious influences they may entirely lose it.

Production of Toxin for Immunization of Horses.—Following is the method of handling stock cultures and of producing toxin, modified by Wilcox[1] in our laboratory from the method used by Anderson and Leake.[2]

Stock Cultures.—The stock cultures are grown on a semisolid agar medium neutral to phenolphthalein.

To transfer cultures, one of the semisolid agar cultures is melted and 1 c.c. is added to a freshly melted semisolid agar tube, at least ten subcultures being thus made from one stock culture. After inoculation, the tubes are cooled, the plugs immersed in paraffin and the tubes incubated at 37° C. After one week's incubation the cultures are stored in the ice-chest, where they may be kept for six months without affecting their ability to produce toxin.

Preliminary Cultivation.—Fill potato tubes with about 40 c.c. of the toxin broth (Chapter IV) sterilize for one and one-half hours on the first day and one hour on the second day. These tubes may be kept for two weeks, up to which time they will give satisfactory growth. To make the first transfer for the preliminary cultivation add 8 c.c. of the melted semisolid agar stock culture of the B. tetani to two tubes of glucose broth from which the air has been previously expelled by heating in the Arnold for fifteen to twenty minutes, and which have been cooled down to about 50° C. These tubes are incubated for twenty-four hours and the next day, two freshly heated tubes of broth are inoculated with 5 c.c. of the glucose broth cultures planted the previous day. On the third day determine the number of flasks that are to be inoculated and inoculate as many freshly heated glucose broth tubes from the second glucose broth generation as there are flasks. Anderson calls for at least six or seven generations in the glucose broth before the inoculation of the toxin broth, but at the Research Laboratory it has been found that three generations, or even two if need be, are sufficient for obtaining a toxicity of 1 to 25,000.

[1] Jour. Bact., 1916, 1, 333.
[2] Jour. Med. Res., 1915, 33, 235.

Inoculation of Toxin Broth.—The flasks of toxin broth after the second sterilization in the Arnold are ready for inoculation. The broth may be cooled down to 55° to 60° C. by allowing the flasks to stand at room temperature, or in a more rapid way by placing the hot flasks in a large sink, to which cool and then cold, water is added until the lower portions of the flasks are covered. When the bottoms of the flasks are cool to the hand, the portions above the water being still very hot, the inoculation may be made as follows:

The plugs are carefully removed, the necks flamed and the plugs replaced. In a similar way, the mouths of the culture tubes are sterilized and then, partly removing the plug of a flask the contents of a potato tube are poured rapidly into a flask. If one prefers, the broth culture may be transferred by using a pipette but the former method has been used here without subsequent contamination and found very satisfactory. After inoculation, the flasks are incubated for fifteen days at 36° to 37° C., care being taken to exclude all light from them.

The flasks at the end of twenty-four to forty-eight hours show a diffuse cloudiness with the formation of gas bubbles on the surface of the broth. Toward the end of two weeks the gas bubbles usually disappear, while the cloudiness persists and a light precipitate forms at the bottom of the flasks. If it is not convenient to filter on the fifteenth day, the cultures may be kept in the incubator until the twentieth day without a loss of toxicity, but from the twentieth day to the twenty-fifth day the toxin loses about 20 per cent. in potency.

Filtration.—All glassware, filters, etc., should be neutral to phenolphthalein and the greatest care should be taken to exclude light, either direct or indirect, by darkening the room and by covering the filtering apparatus with dark cloths, ordinary black cambric being used.

The broth cultures are first passed through Buchner filters about 8 inches in diameter, which have been packed with a layer of finely shredded paper pulp 0.25 inch in thickness. It is of importance that the pulp should be so well packed that the filtrate is absolutely clear, otherwise it will clog the Berkefeld filter. The first filtrate, about 200 c.c., which passes through the Buchner is discarded, as it contains a considerable amount of water from the pulp, and then the filtering of the toxin may proceed. If, after passing 8 to 14 liters through the pulp, the filtrate begins to appear cloudy, the pulp must be discarded and the Buchner repacked. The clear filtrate is then passed through a sterile Berkefeld filter and 10 per cent. of a 5 per cent. solution of carbolic acid solution is added to the toxin which is now placed in the ice-chest, ready for testing its potency.

Potency Test.—Two 350-gram guinea-pigs are inoculated subcutaneously over the abdomen with 1 c.c. of a dilution of 1 to 15,000 and 1 to 25,000 of the toxin respectively. If the toxin has a potency of 25,000 the pig receiving the 15,000 dilution will die on the second to third day and the pig receiving the 25,000 dilution should die on the fourth day. If both pigs die with symptoms of tetanus before the fourth day, the toxin is stronger than 25,000 and a higher dilution should be tested.

Action of Tetanus Toxin in the Body.—After the absorption of the poison there is a lapse of time before any effects are noticed. In experimental animals with an enormous amount, such as 90,000 fatal doses, this is about nine hours; with 30,000, ten; with 3000, twelve; with 300, twenty hours; with ten fatal doses, thirty-six to forty-eight hours; with two fatal doses, two or three days; with one fatal dose, four to seven days. Less than a fatal dose will produce local symptoms. The parts first to be affected with tetanus in these animals are usually the muscles lying in the vicinity of the inoculation—for instance, the hindfoot of a mouse inoculated on that leg is first affected, then the tail, the

other foot, the back and chest muscles on both sides and the forelegs, until finally there is a general tetanus of the entire body. In mild cases, or when a dose too small to be fatal has been received, the tetanic spasm may remain confined to the muscles adjacent to the point of inoculation or infection. The symptoms following a fatal dose of toxin vary greatly with the method of injection. Intraperitoneal injection is followed by symptoms which can hardly be distinguished from those due to many other poisons. Injection into the brain is followed by restlessness and epileptiform convulsions. In man the first symptoms are usually those of a contraction of the muscles of the lower jaw and then those of the neck. In about one-third of the human cases, the muscles about the site of inoculation are first affected.

Theories as to the Methods by which the Toxin Produces its Effects.—Gamprecht and Stintzing concluded from their experiments that the toxin from the wound passed to the central nervous system partly directly by the peri- and endoneural lymph spaces of the nerves which directly connected with subdural space, and partly indirectly from the blood. The local tetanus they considered as due to the contact of the poison with the motor end-plates. The experiments of Meyer and Ransom and of Marie and Morax have proved to them that the poison is transported to the central nervous system by the way of the motor nerves—and by no other channel. These authors thought that they had shown that the essential element for the absorption and transportation of the toxin is not the lymph channels, but the axis-cylinder, the intramuscular endings of which the toxin penetrates. The poison is taken up quite rapidly. Marie and Morax were able to demonstrate the poison in the corresponding nerve trunk (sciatic) one and a half hours after the injection. Absorption, however, and conduction are dependent to a large extent on the nerves being intact. A nerve cut across takes very much longer to take up the poison (about twenty-four hours), and a degenerated nerve takes up no poison whatever. In other words, we see that section of the nerve prevents the absorption of the poison by way of the nerve channels. The poison which passed through the general lymph channels to the blood was partly returned to the tissue fluids throughout the body and taken up by nerve endings and thus produced general tetanus.

According to Meyer and Ransom, the reason why the sensory nerves do not play any rôle in the conduction of the poison lies in the presence of the spinal ganglion, which places a bar to the advance of the poison. Injections of toxin into the posterior root lead to a tetanus dolorosus, which is characterized by strictly localized sensitiveness to pain.

Ascending centripetally along the motor paths, the poison reaches the motor spinal ganglia on the side of inoculation; then it affects the ganglia of the opposite side, making them hypersensitive. The visible result of this is the highly increased muscle tonus—*i. e.*, rigidity. If the supply continues, the toxin next affects the nearest sensory apparatus; there is an increase in the reflexes, but only when the affected portion is irritated. In the further course of the poisoning the toxin as it ascends continues to affect more and more motor centers, and also the neighboring sensory apparatus. This leads to spasm of all the striated muscles and general reflex tetanus. Zupnik showed that local tetanus did not develop when toxin was injected where it did not come in contact with muscle. He believed, therefore, that the muscle spasm was due to direct action of the toxin on the muscle fibers.

Field in our laboratory has shown that not only tetanus toxin, but diphtheria toxin and inert colloids can be demonstrated in the sciatic nerves after they

have been injected subcutaneously or intramuscularly, and after varying periods may be found in the spinal cord. He believes that the toxins are absorbed by way of the lymphatics of the nerves and not by way of the axis-cylinder.

A later experiment of Cernovodeanu and Henni tends to confirm this contention. They ligated all the muscles and bloodvessels in a guinea-pig's leg, leaving intact only the sciatic nerve, skin and bone, and then injected a large amount of tetanus toxin below the point of ligation. The animals in which this was done never developed tetanus. In this case there was only a very slight flow of lymph into the ligated area, and so there could be only a slight flow up the nerve.

If the toxin gets into the blood the only path of absorption to the central nervous system is still by way of the motor-nerve tracts. There seems to be no other direct path, as, for example, by means of the bloodvessels supplying the central nervous system. Even after introducing the poison into the subarachnoid space, owing to the passage of the poison into the blood, there is a general poisoning and not a cerebral tetanus. This at least is the case if care has been taken during the operation to avoid injuring the brain mechanically. A very much smaller amount of toxin is required to produce fatal tetanus if it is injected into a nerve than if it is injected into the blood.

The Union of Toxin with the Gray Matter of the Brain and Spinal Cord.—This union is a loose one and the toxin can be partially freed from its union by action of proteolytic ferments. A number of different elements of the cell substances seem to have this power of binding the toxins. Heating to 65° C. for ten minutes destroys the capacity to fix toxin. These brain substances which unite with toxin are certainly not of the nature of antitoxin, and the brain cells if they produce antitoxin at all certainly share the power with other cells that have no power to bind toxin. Lecithin compounds are undoubtedly concerned in the mechanism of the binding of tetanus toxin by the nerve cells.

Presence of Tetanus Toxin in the Blood.—The blood during the first four days of the disease usually contains toxin. After that time antitoxin usually develops and soon makes the blood antitoxic. In St. Louis some years ago the serum of a horse dying of tetanus was given by accident in doses of 5 to 10 c.c. to a number of children, with the development of fatal tetanus. In this connection Bolton and Fisch showed by a series of experiments that much toxin might accumulate in the serum before symptoms become marked.

Tetanus Antitoxin.—Behring and Kitasato were the first to show the protective and curative effects of the blood serum of immunized animals. It was found that animals could be protected from tetanus infection by the previous or simultaneous injection of tetanus antitoxin, provided that such antitoxin serum was obtained from a thoroughly immunized animal. From this it was assumed that the same result could be produced in natural tetanus in man. Unfortunately, however, the conditions in the natural disease are very much less favorable, inasmuch as treatment is usually commenced not shortly after the infection has taken place, but some hours after the appearance of tetanic symptoms, when the poison has already attacked the cells of the central nervous system.

The Production of Tetanus Antitoxin for Therapeutic Purposes.—(See Chapter VIII.) While tetanus antitoxin has proved most efficacious in the prevention of tetanus, its employment as a curative agent has been much less successful. This failure to produce more uniformly good results is due chiefly to its too late administration. Insufficient dosage and the use of the subcutaneous method have also been important factors. While the subcutaneous use of antitoxin, which at first was the usual method employed, may prove of value in large doses given within the first few hours after the onset of symptoms, nevertheless, it frequently fails because of slow absorption and the time required to reach the tissues of the central nervous system. In the light of our present knowledge, this method can be justified only by an inability of the physician, for one reason or another, to give an intravenous or an intraspinal injection. The latter method is being more and more recommended and when employed before the disease has made too much headway has given much better results than the subcutaneous or even the intravenous.

From time to time since 1903 single cases or small series of cases have been reported in which the antitoxin has been given intraspinally. Thus Neugebauer[1] in 1905 reported 43 cases from Continental and American sources with a mortality of 22, or 51 per cent., and also 3 patients treated by him of whom 2 recovered. Many of these patients received also intravenous or subcutaneous injections or both. The results seemed to some observers to be better than when other methods were used without intraspinal dosage, but on the whole, this method of treatment has not made much headway. Experimental proof of the greater value of the intraspinal method has not until very recently been at all convincing. Permin, of Copenhagen, working on rabbits and dogs, showed that local tetanus could be prevented by the simultaneous injection of tetanus toxin intramuscularly and a dose of antitoxin given into the spine, whereas with the same dose of antitoxin given intravenously, local tetanus occurred. Four hours after the giving of the toxin alone neither method was efficacious in preventing the occurrence of local tetanus, and when nine hours were allowed to elapse between the giving of toxin and antitoxin, the animals could not be saved.

The following series of experiments were undertaken by Nicoll and us to determine to what extent tetanus antitoxin given in the spine has greater curative power, when the disease is actually established, than when given in the circulation. The comparative ineffectiveness of subcutaneous injections of tetanus antitoxin in developed cases is incidentally brought out. The use of guinea-pigs for this purpose has not been recorded so far as we know. They possess, however, a marked susceptibility to tetanus together, with comparative freedom from intercurrent diseases. The toxin in each case was inoculated intramuscularly into the thigh.

[1] Wiener klin. Wchnschr., 1905, **18**, 450.

ADMINISTRATION AND RESULT OF TETANUS ANTITOXIN.

No.	Weight. gms.	Condition of leg.	Method.	Amount. in units.	Result.
116	290	Fairly stiff	Control	...	D 3 days.
42	310	Fairly stiff	Control	...	D 3 days.
296	250	Slightly stiff	Heart	100	D 8 days.
227	275	Fairly stiff	Heart	100	D 4 days.
399	300	Fairly stiff	Heart	100	D 5 days.
216	255	Slightly stiff	Nerve	200	D 4 days.
287	255	Fairly stiff	Nerve	200	D 3 days.
289	280	Fairly stiff	Nerve	200	D 3 days.
306	285	Slightly stiff	Nerve	200	D 3 days.
59	255	Stiff	Spine	10	Discharged; normal.
304	275	Fairly stiff	Spine	10	Discharged; drags leg.
321	320	Fairly stiff	Spine	10	Discharged; drags leg.

SIX GIVEN ANTITOXIN TWENTY-TWO AND A HALF TO TWENTY-THREE HOURS AFTER INOCULATION OF TOXIN.

102	300	Stiff	Heart	100	D 5 days.
10	325	Stiff	Heart	200	D 4 days.
272	350	Stiff	Heart	200	D 4 days.
263	285	Stiff	Spine	50	Discharged; drags leg.
123	325	Stiff	Spine	50	D 5 days.
294	350	Stiff	Spine	50	Discharged; drags leg.

This would seem absolutely conclusive of the superiority of the intraspinal method of giving antitoxin over the intravenous method. It will be noted that only those animals receiving the antitoxin in the spine were able to survive. This result is the more striking since the amount of antitoxin given them was only a fractional part of that given in the circulation. An attempt was made to give the antitoxin to four guinea-pigs intraneurally. Under an anesthetic the sciatic nerve of the affected limb was cut down upon and freed from the surrounding tissues, as much antitoxin as could be introduced into the nerve sheath injected, and the remainder intramuscularly directly along the course of the nerve. Owing to the small caliber of the nerve, most of the antitoxin passed to the deep intramuscular tissues. The animals lived no longer than those receiving subcutaneous injections.

In all of these experiments no guinea-pigs were discharged from observation until there was absolutely satisfactory evidence that the disease had long ceased to make any progress. While a number of the animals, perhaps most, on being discharged exhibited more or less stiffness in the inoculated leg lasting for weeks or even months, they were nevertheless in the best of health in other respects. This condition appears to be identical with that seen in human tetanus, in which the stiffness in different groups of muscles is often very prolonged even when the patients are perfectly convalescent.

Several years ago we were able to obtain records of twenty-four con-

secutive clinical cases of tetanus in which an early intraspinal injection of antitoxin was given and eighteen patients recovered. In all of them in addition to the antitoxin used intraspinally, larger amounts were also given by other methods.

Treatment of a Developed Case.—It is of vital importance, in a case of developed tetanus, that a large dose of antitoxin be administered at the earliest possible moment and in such a manner as to reach the spinal nerve centers as quickly as possible.

The first injection should, therefore, be made into the spinal canal.

A lumbar puncture having been performed in the usual way, preferably under an anesthetic, 5000 to 10,000 units of antitoxin, heated to body temperature, are allowed to run into the spinal canal by gravity. In order that the antitoxin may be distributed throughout the length of the cord, it should, if necessary, be diluted with sterile saline to a volume of at least 5 c.c. In case of a "dry tap," which has been reported on good authority as occasionally occurring in this disease, this amount should not be exceeded, but when an abundance of spinal fluid is obtained the intraspinal dose should be a little less than that of the fluid withdrawn. An intravenous injection of 10,000 units should be given at the same time in order to render the blood highly antitoxic at once. The intraspinal injection may be repeated in twenty-four hours and again in forty-eight hours. An intramuscular injection of 5000 units may also be given on the third and fourth day to sustain the antitoxic strength of the blood. If, for any reason, the attending physician is not able to give an intraspinal injection, an intravenous dose of 15,000 to 20,000 units should be given. If this, too, is impossible, rather than delay, a dose of 30,000 units should be given intramuscularly, several muscles being used, and arrangements immediately made to give an intraspinal and intravenous dose at the earliest possible moment.

The use of antitoxin does not do away with the necessity for thorough surgical treatment of the wound, if it has not already healed. The patient should be protected as carefully as possible from noise, excessive light, draughts, jars and other forms of irritation. Sedatives of the chloral type are well borne in large doses and should be pushed to the limits of safety. Heart stimulants are often indicated. Feeding the patient is frequently a very difficult problem, requiring the utmost patience and ingenuity.

Immunization against Tetanus.—For immunization in case of blank cartridge and other wounds, especially crushed wounds into which dirt has been ground and punctured wounds made by rusty nails where infection with tetanus bacilli is feared, a preventive injection of 1500 to 3000 units is strongly advised. In severe crushed wounds a second injection of 3000 units should be given at the end of a week to prolong the immunity, and if the wound has not healed at the end of the second week a third injection is advised.

Results of the Use of Antitoxin for Immunization.—The striking results which have been obtained, both in human and in veterinary

practice, with the prophylactic injection of tetanus antitoxin, would seem to warrant the treating of patients with immunizing doses of serum—at least in neighborhoods where tetanus is not uncommon—when the lacerated and dirty condition of their wounds may indicate the possibility of a tetanus infection.

Splendid results have followed this practice in many places. It is the custom at many dispensaries in New York City and elsewhere to immunize all Fourth-of-July wounds by injecting in slight injuries 1000 units and in severe ones 2000 to 3000 units. Almost none of these has ever developed tetanus. Even the few cases of human tetanus reported as occurring after single injections of antitoxin prove the value of immunizing injections, for the mortality was low. They teach, however, that where tetanus infection is suspected the antitoxic serum should be given a second and even a third time at intervals of seven days. In the European armies it was compulsory to give a serum injection to every wounded soldier at the first possible moment after the injury. A second injection was given ten days later. After these regulations were adopted there have been almost no cases of tetanus.

Persistence of Antitoxin in the Blood.—Ransom has clearly shown that the tetanus antitoxin, whether directly injected or whether produced in the body, is eliminated at the same rate from the blood of an animal, provided that the serum was from an animal of the same species. If from a different species it is much more quickly eliminated.

The same author found some interesting facts in testing the antitoxic values of the serum of an immunized mare, of its foal, and of the milk. The foal's serum was one-third the strength of the mare's and one hundred and fifty times that of the mare's milk. In two months the mare's serum lost two-thirds in antitoxic strength, the foal's five-sixths, and the milk one-half.

Rapidity of Absorption and Loss of Tetanus Antitoxin from Tissues.—The absorption of antitoxin administered subcutaneously takes place rather slowly. In his animal experiments Knorr found the maximum quantity in the blood only after twenty-four to forty-eight hours. From that time on the amount again steadily decreased, so that by the sixth day only one-third the optimum quantity was present. By the twelfth day only one-fiftieth and at the end of three weeks no antitoxin whatever could be demonstrated. We injected a volunteer laboratory assistant with 10,000 units of tetanus antitoxin subcutaneously. The blood antitoxic strength was found to be as follows: Eighteen hours, 0.6 unit; twenty-four hours, 0.8; forty-eight hours, 1; seventy-two hours, 1; one hundred and forty-eight hours, 0.7. The two important facts to be noted are the slow absorption of antitoxin from the subcutaneous tissues and its long retention in the blood. The two charts showing the absorption and disappearance of diphtheria antitoxin apply equally to tetanus antitoxin (see Figs. 77 and 78, pages 192 and 193).

When injected intravenously the antitoxin very quickly passes into the lymph. Ransom, in 1901, was able to demonstrate it in the thoracic duct of a dog fifteen minutes after intravenous injection. Only after

very massive intravenous doses and a considerable interval of time are small traces found in the cerebrospinal fluid. This is the reason that passively and actively immunized animals become tetanic if the poison is injected directly into the central nervous system or into a peripheral nerve. Antitoxin injected subdurally passes almost entirely over into the blood within twenty-four hours.

So long as the toxin circulates in the blood it is neutralized by antitoxin in about the same proportion as in test-tube experiments. By means of intravenous injections of antitoxin Ransom was able to render the lymph free from toxin in a very few minutes. According to Marie and Morax, toxin injected into the muscles is already demonstrable in the nerve tissue at the end of one and a half hours—*i. e.*, it has already entered the channel, where it is reached with difficulty by the antitoxin. Donitz injected various rabbits intravenously, each with 1 c.c. of a toxin solution containing twelve fatal doses. Thereupon he determined the dose of antitoxin which, when intravenously given, would neutralize this poison after various intervals of time The antitoxin was of such a strength that in test-tube experiment 1 c.c. of a 1 to 2000 solution just neutralized the amount of toxin employed. He found that at the end of two minutes double the dose required *in vitro* would still neutralize the poison; at the end of four minutes about four times the dose was required, and at the end of eight minutes ten times. When one hour had been allowed to elapse forty times the original dose just sufficed to protect the animal from death, but not from sickness.

Agglutinative Types.—Tulloch[1] has reported four serological agglutinative types by the absorption of agglutinins. Type I is obtained the most frequently and from the less severe cases. Type IV is very infrequent. Tetanospasmin does not appear to be specific to any one type; an antitoxin neutralizes the toxin of each type equally well.

Differentiation between Tetanus and Tetanus-like Bacilli.—The identification of the bacillus of tetanus is, generally speaking, not difficult, inasmuch as animal inoculation affords a sure test of the specific organism. No other microörganism known produces effects similar to the tetanus bacillus, nor is any other neutralized by tetanus antitoxin. The other characteristics also of this bacillus are usually distinctive, though microscopic examination alone cannot be depended on to make a differential diagnosis. Difficulty arises when other anaërobic, or aërobic bacilli, almost morphologically identical with the tetanus bacillus, are encountered which are non-pathogenic, such as the Bacillus pseudotetanicus (anaërobius), and the Bacillus pseudotetanicus (aërobius). It is possible, however, that both these bacilli, when characteristic in cultures, are only varieties of the tetanus bacillus, which, under unfavorable conditions of growth, have lost their virulence. These non-virulent types do not, as a rule, have spores absolutely at their ends and the spores themselves are usually more ovoid than those in the true tetanus bacilli.

[1] Jour. Hyg., 1919–1920, **18**, 103.

Methods of Examination in a Case of Tetanus.—(*a*) *Microscopic.*—From every wound or point of suppuration film preparations should be made and stained with the usual dyes. The typical spore-bearing forms are looked for, but are usually not found. At the same time other bacteria are noted if present.

(*b*) *Isolation of Pure Culture.*—The growth of the tetanus bacillus in the animal body is comparatively scanty, and is usually associated with that of other bacteria; hence the organism is difficult to obtain in pure culture. It is still more difficult to obtain it from infected material other than the animal body. The method of procedure which is most successful, consists in inoculating the tetanus-bearing material (pus or tissue from the inoculation wound, cartridge wads, etc.), into tubes of freshly sterilized slightly alkaline nutrient agar, chopped meat media, or fermentation tubes of glucose bouillon to each of which a piece of fresh sterile tissue has been added, and incubating at 37° C. After the tetanus spores have formed as shown in microscopic preparations from the sediment, heat for one-half hour at 80° C. to destroy the associated bacteria and subinoculate in broth and make plates. If the tetanus bacilli are the only spore-bearing bacteria present, pure cultures are readily obtained; when other spore-bearing anaërobic bacteria are present, the isolation of a pure culture may be a matter of difficulty, but even then the presence of tetanus toxin in the culture fluid, shown by the inoculation of animals, will indicate the presence of tetanus bacilli.

(*c*) *Inoculation.*—A salt solution emulsion of material from the wound is inoculated into mice or guinea-pigs subcutaneously. Successful results may be obtained, if the bacilli or their spores are present.

BACILLUS BOTULINUS (CLOSTRIDIUM BOTULINUM).

This bacillus, while not pathogenic for man as an infectious organism, is, as first shown by Van Ermengen (1896), the causative agent in a characteristic type of food poisoning, the symptoms being due to the toxin produced by the growth of this bacillus in foods. The clinical symptoms of this type of food poisoning are neuro-paralytic in character. There are secretory disturbances, as increase or suppression of the salivary secretions or a thick secretion of mucus in the mouth and pharynx. Disturbances of accommodation, ptosis, double vision and dysphagia are the common motor symptoms. Obstinate constipation and retention of urine as well as disturbances in heart action and respiration occur. Fever is absent. Death is not uncommon and is due to respiratory paralysis. Graham and Brueckner[1] have recently reported results indicating that the toxin of this bacillus or similar bacilli is the causative agent in some outbreaks of forage poisoning or so-called cerebrospinal meningitis in horses. The botulinus bacillus is also the causative agent of "limber neck" of chickens.[2]

[1] Jour. Bact., 1919, **4**, 1.

[2] Bengston: Pub. Health Rep., 1923, **38**, 340.

Morphology.—The bacilli are large, 4 to 6μ long and 0.9 to 1.2μ wide, with somewhat rounded ends. They are slightly motile (4 to 8 flagella), Gram-positive, and have oval spores, usually terminal or subterminal. Short chains may be produced.

Biology.—It is anaërobic, growing best at 22° to 25° C., at which temperatures spores are more readily produced. Gelatin is liquefied and glucose fermented with the production of acid and gas. Results of the fermentation of other sugars differ according to different investigators. A butyric acid odor develops in cultures. The colonies on gelatin are yellowish, translucent, coarsely granular, the granules moving slowly when liquefaction begins. The older colonies are brownish, more opaque and show fine thorn-like extensions.

Resistance.—The spores were reported as being moderately resistant to heat, but the work of Esty and Meyer[1] shows that they vary much in their resistance. The maximum resistance observed with Types A and B is ten minutes at 115° C. and three hundred and thirty minutes at 100° C., moist heat. Type C is considerably less resistant.

Toxin Production.—A thermolabile extracellular toxin is produced in glucose-broth cultures. Guinea-pigs, rabbits, mice, cats and monkeys are susceptible to the toxin and succumb not only to injection but also when the toxin is given by mouth. As little as 0.0001 c.c. may produce symptoms. After an incubation period, dyspnea, convulsions and paralysis occur and death is due to respiratory paralysis. The paralyses have been attributed to degeneration of the ganglion cells of the anterior horn and of the bulbar centers. According to Dickson[2] this is secondary to disturbances in blood supply due to thrombosis associated with meningeal hemorrhage. Edmunds and Long[3] have shown that the paralysis is due to the action of the toxin on the nerve endings of the motor nerves. Three types of toxin have been demonstrated. The first to isolate the second type (Type B) was Nevin.[4] Bengston[5] has found a third type among cultures from fly larvae and "limber neck" cases.

The question as to whether toxin-free spores are pathogenic has been much studied. Coleman and Meyers[6] sum up the work done, adding original work and come to the conclusion that with massive doses animals may be infected with spores which germinate in the body.

Antitoxin.—An antitoxin has been prepared for each of the types. This is efficacious in preventing death in experimental animals if used early enough. The results in man have been disappointing. This may be due to the early development of symptoms, at which time antitoxin is not usually immediately available. Death is due to respiratory

[1] Jour. Inf. Dis., 1922, **31**, 650.

[2] See Botulism—A Clinical and Experimental Study, Monograph VIII, Roch. Inst. Med. Res., 1918.

[3] Jour. Am. Med. Assn., 1923, **81**, 542.

[4] Reported in 1915 at Society of Am. Bact. and published in Jour. Inf. Dis., 1921, **28**, 226. The strain had been sent to many laboratories in the meantime.

[5] Pub. Health Rep., 1923, **38**, 340.

[6] Jour. Inf. Dis., 1922, **31**, 622; Am. Jour. Hyg., 1929, **14**, 47.

paralysis and the antitoxin must protect the nerve endings from serious injury if this is to be prevented.

Foods Involved.—Meat and meat preparations, such as canned or common pickled meats and sausages are offending foods. Until recently it was thought that preserved or canned fruits or vegetable products were not factors. Dickson has shown that the toxin develops in a medium of peas or beans. A considerable number of cases associated with the consumption of canned vegetables have been reported. Ripe olives have caused a number of outbreaks. All these foods have one thing in common, they are prepared weeks or months before they are consumed. The sources of contamination may be many since it has been recently shown that the spores may be isolated from widely distributed soils (Meyer and Geiger, and Meyer and others[1]) and from feces of different animals. In any lot of canned goods some containers may be toxic and others not.

Diagnosis of Botulism.—The diagnosis usually rests on the clinical manifestations. Verification may be attempted by examination of the suspected food. Direct intraperitoneal inoculation into white mice or guinea-pigs with the food suspected of containing the toxin may be made. Duplicate animals may be injected also with the "type" antitoxins as a further proof of the nature and of the type of toxin. For further tests and attempts to obtain pure cultures the following procedure may be carried out. A thick emulsion of the food is made in saline, and from it pour plates or "shake" tubes are made, and fermentation tubes (with tissue) are inoculated. Glucose agar is usually employed. A portion of the suspension can be heated (60° C., for one-half hour) and similarly inoculated. From the growth in the fermentation tube, heated and unheated samples are used to inoculate plates or "shake tubes." The pure cultures obtained are then tested for toxin production by animal inoculation. Saline extracts of the suspected food, if they produce characteristic symptoms in experimental animals, confirm the clinical diagnosis in man. The type is determined by demonstrating the neutralizing action of the type antitoxins on the toxin in the food or the toxin produced by isolated bacillus. Intoxication is more common from foods prepared in the west where Type A predominates, than in the east where Type B is more frequently found.

Prophylaxis.—A rancid butter odor may be present in contaminated foods, but it may be slight and not recognized. Although the toxin is destroyed by heating to 80° C. in ten minutes, all canned material should be heated to boiling before use. Ten or fifteen minutes' boiling is recommended to insure thorough penetration of the heat. Cleanliness in preparation is an aid in preventing contamination and also in limiting the numbers of associated bacteria which, by their growth, would aid in establishing anaërobic conditions. Brine for pickling should contain at least 10 per cent. of salt, as this concentration prevents the growth of the bacilli. Incomplete sterilization is the source of danger with canned goods.

[1] Pub. Health Rep., 1921, **36**, 1313; Jour. Inf. Dis., 1922, **31**, 501, 540.

GASEOUS AND PHLEGMONOUS GANGRENE AND THE ASSOCIATED ANAËROBES.

The infection through wounds with the gas-forming anaërobes occurs seldom in humans except during times of war, or following untreated dirty wounds. In such infections pyogenic aërobes are also frequently present so the picture is often a complex one. It was earlier thought that "B. welchii" was the common cause of gaseous gangrene in man, but the studies made during the Great War have shown that, while the B. welchii group is most frequently found, it is seldom alone. It is accompanied either by forms non-pathogenic in themselves, like B. sporogenes, but having the power to enhance the pathogenicity of B. welchii, or by forms that are more or less pathogenic than B. welchii. The following table from Weinberg and Seguin[1] shows the relative frequency of single and mixed infections with these forms in war wounds.

Name of anaërobe.	Number of deaths due to anaërobes.			Total number of cases showing anaërobe.
	Single species.	Associated with an anaërobe less pathogenic.	Total.	
B. welchii	9	10	19	91
B. œdematiens	3	9	12	33
Veb. septique	1	3	4	12
B. fallax	..	1	1	26
B. welchii + B. œdematiens			2	
B. welchii + Vib. septique + B. fallax			1	

A classification of the chief war wound anaërobes was also given by McIntosh.[2]

The part the laboratory played in an early diagnosis and in aids for treatment during the World War may be summarized as follows:

The number and kinds of bacteria found in war wounds depend chiefly upon the time elapsing between the hour when the patient is wounded and the hour when the examination of the wound is made. Practically no organisms can be demonstrated in cultures until at least four hours or in smears until at least six hours after the wound is made. After that, several varieties of both anaërobic and aërobic bacteria develop quickly in untreated infected wounds.

Many aërobes have been found in infections in war wounds; for example, staphylococci, St. hemolyticus and viridans, "enterococcus," B. pyocyaneus, B. proteus, B. coli, B. diphtheriæ, diphtheroid bacteria. These with the exception of hemolytic streptococci are probably secondaries and aid the development of the anaërobes.

There are two practical reasons for making a bacteriological examination of an open wound. The first is to determine the type of anaërobe

[1] La gangrène gazeuse, 1918, Paris.
[2] Publication of Medical Research Com., London, 1917, p. 74.

present in order to give specific serum. Serums which are specific have been produced against Vibrion septique, B. welchii, B. œdematiens respectively (see Part III), and Sacquepée[1] recommends the following procedure for the differentiation.

In each of four test-tubes is placed 1 c.c. of the macerated gangrenous tissue and to three tubes, respectively, is added 1 c.c. of each of the three antiserums. After half an hour's incubation the contents of each tube is injected respectively into one of four guinea-pigs, according to Sacquepée. The one protected by the serum shows no reaction; the others die. They usually become sick in from six to twelve hours.

The second reason for making a bacteriological diagnosis is in order to inform the surgeon as to the possibility and the time for performing a primary, delayed primary or a secondary closure. The bacteriological examination of recent injuries, to be of most aid to the surgeon (according to Beebe[2]), should be made after the primary operation as follows:

A few strands of silkworm gut or horse-hair should be placed at the most dependent part of the wound. At the first dressing, twenty-four hours later, these should be removed and from the serum that exudes the bacteriologist should make his cultures and smears. The examination of open wounds should be made from material curetted from the deeper pockets.

If the count in the stained spreads shows only one bacterium in two fields, or less, for two successive counts and the cultures show no hemolyzing cocci and very few anaërobic bacilli, the wound may be closed. If hemolytic streptococci have been shown to be present, the wound should not be closed until several repeated cultures show the absence of hemolytic streptococci. Considerable numbers of staphylococci and of bacilli of the colon group sometimes prevent primary union. The counting of the number of bacteria in the coverglass is not an accurate procedure, but suffices for guidance when controlled by cultures.

THE MALIGNANT EDEMA BACILLUS OR VIBRION SEPTIQUE. (CLOSTRIDIUM ŒDEMATIS-MALIGNI).

Bacilli of this group are widely distributed, being found in the superficial layers of the soil, in putrefying substances, in foul water. This bacillus was discovered (1877) by Pasteur in animals after infection with putrid flesh, and named by him "vibrion septique." He did not obtain it in pure culture. Koch and Gaffky (1881) carefully studied a similar microörganism, described it in detail, and gave it the name "Bacillus œdematis maligni" (Fig. 157). It is considered that the bacillus of Ghon and Sachs and Bacillus III of Van Hibler are identical with this organism. Authors disagree as to whether Koch's bacillus is the same as Pasteur's. Some think Koch was dealing with B. sporogenes.

Morphology.—The edema bacillus is a rod of from 0.8μ to 1μ in width, and of very varying length, from 2μ to 10μ or more, according to the conditions of its cultivation and growth. It is usually found in pairs, joined end to end, but may occur in chains or long filaments. It

[1] Sacquepée and de la Vergne: de l'Acad. de Méd., 1919, 81, 504.
[2] Wound Bacteriology, War Medicine, January, 1919, p. 1023.

is motile, and does not produce a capsule. It forms spores which are situated in or near the middle of the body of the rods, exceptionally near the ends. The spores vary in length and are oval in form, being often of greater diameter than the bacilli, to which they give a more or less oval shape.

The bacilli *stain* readily by the usual anilin colors employed and are Gram-amphophile.

Biology.—An obligate anaërobe growing best on or in albuminous media but also growing well on ordinary media especially if available carbohydrates are present. Growth occurs at 20° C., but is more rapid and abundant at 37° C.

Growth on Agar.—On dextrose agar plates the colonies appear as dull, whitish points, irregular in outline, and when examined under a low-power lens are seen to be composed of a dense network of interlacing threads, radiating irregularly from the center toward the periphery.

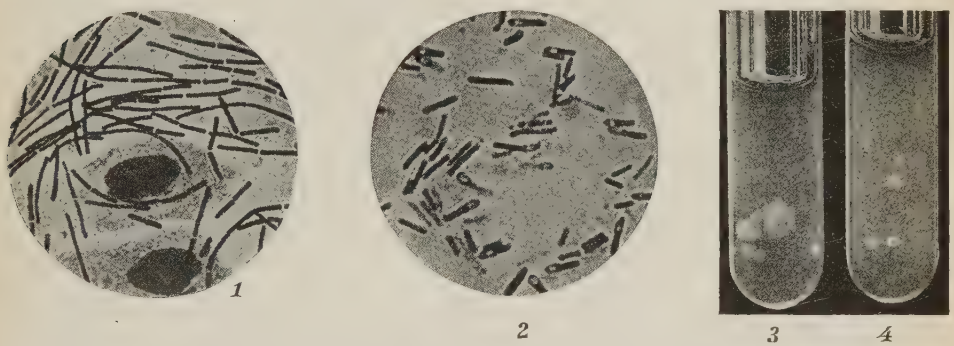


FIG. 157.—Bacilli of malignant edema. 1, bacilli; 2, with spores; 3 and 4, deep colonies in dextrose nutrient agar. (Kolle and Wassermann.)

Growth in Gelatin.—The colonies are similar to those on agar, a liquefied zone developing after several days.

Resistance.—The spores are very resistant and because of this the soil remains infected.

Other Media.—Milk is coagulated but not digested. Blood serum is not liquefied. Most of the test sugars, with the exception of sucrose are fermented with the production of acid and gas.

Pathogenicity.—Malignant edema is ordinarily confined to domestic animals, horse, sheep, cattle and swine. It follows the contamination of wounds with infected soil or other infectious material; and also occurs as a complication of surgical operations. It is therefore a frequent contaminant in war wounds. The depth of the wound as well as the introduction of foreign particles and other bacteria are factors in infection, as the inoculation of washed spores frequently fails to produce infection. An extensive hemorrhagic edema of the subcutaneous tissues develops from the site of the wound. The serous effusion is frothy from gas production and has a foul odor. As a rule in the larger animals the bacilli do not invade the blood until after

death. The bacillus is pathogenic for the smaller laboratory animals, rabbit, guinea-pig, mouse, a septicemia developing as well as the local edema.

Immunity.—Recovery from infection is followed by immunity. Weinberg and Seguin[1] and others have demonstrated a toxin and have produced a serum effective in helping to prevent gaseous gangrene.

Agglutinins.—Three agglutinative groups have been demonstrated by Robertson,[2] the toxins of which are all neutralized by a single antitoxin. In this work Robertson also showed that her strains of B. chauvei were distinctly different, culturally and serologically, from her strains of Vib. septique. She did not agree, therefore, with Nicolle[3] in concluding that they are the same species.

BACILLUS OF SYMPTOMATIC ANTHRAX (CLOSTRIDIUM CHAUVEI).

The bacillus of symptomatic anthrax is the cause of the disease in animals—principally cattle and sheep—known as "black leg," "quarter evil," or symptomatic anthrax (rauschbrand, German; charbon symptomatique, French), a disease which is characterized by a peculiar emphysematous swelling of the subcutaneous tissues and muscles, especially over the quarters. Clinically it is sometimes confused with anthrax. Like B. anthracis, it is an inhabitant of the soil and is a large spore-bearing organism, but it grows only under anaërobic conditions and is otherwise distinctly different from B. anthracis. It has not been isolated from wound cultures in man, but some authors consider it identical with Vib. septique (see above).

Morphology.—Bacilli having rounded ends, from 0.5μ to 0.6μ broad and from 3μ to 5μ long, mostly isolated, also occurring in pairs, joined end-to-end, but never growing out into long filaments, as the anthrax bacilli in culture and the bacilli of malignant edema in the bodies of animals are frequently seen to do. The bacilli are actively motile. The spores are elliptic in shape, usually thicker than the bacilli, lying near the middle of the rods. This gives to the bacilli containing spores a somewhat spindle shape. It stains readily with the ordinary dyes and is Gram-amphophile.

Biology.—It is a strict anaërobe, developing at room temperature, better at 37° C. It grows best in albuminous or tissue media and when glucose is present. It ferments this sugar, as well as others except salicin, with the production of gas. Gelatin is liquefied, but coagulated horse serum is not.

Growth on Agar.—The colonies on agar are somewhat more compact than those of malignant edema, but also send out filamentous projections.

Pathogenicity.—The bacillus of symptomatic anthrax is pathogenic for cattle, sheep, goats, guinea-pigs, and mice. The guinea-pig is the

[1] La gangrène gazeuse. Masson et Cie,. Paris, 1918.
[2] Jour. Path. and Bact., 1919–1920, **23**, 153
[3] Ann. de l'Inst. Past., 1915, **29**, 165

most susceptible of test animals, and cultures from the heart blood of inoculated guinea-pigs offer the best method of obtaining pure cultures. When susceptible animals are inoculated subcutaneously with pure cultures of this organism, or with spores attached to a silk thread, or with bits of tissue from the affected parts of another animal dead of the disease, death ensues in from twenty-four to thirty-six hours. At the autopsy a bloody serum is found in the subcutaneous tissues, extending from the point of inoculation over the entire surface of the abdomen, and the muscles present a dark red or black appearance, even more intense in color than in malignant edema, and there is a considerable development of gas. The lymphatic glands are markedly hyperemic.

The disease occurs chiefly in cattle, more rarely in sheep and goats; horses are not attacked spontaneously—*i.e.*, by accidental infection. In man infection has never occurred. The usual mode of natural infection by symptomatic anthrax is through wounds which penetrate not only the skin, but the deep, intercellular tissues; some cases of infection by ingestion have been observed. The pathological findings present the conditions above described as occurring in the experimental animals.

FIG. 158.—Bacilli of symptomatic anthrax showing spores. (After Zettnow.)

Distribution Outside of the Body.—Symptomatic anthrax, like anthrax and malignant edema, is a disease due to soil infection, being confined especially to places over which infected herds of cattle have been pastured. By contamination of deep wounds acquired by animals in infected pastures, the disease is spread. The spores are extremely resistant.

Toxins.—Under favorable conditions extracellular toxins are formed. Injections of the toxin into animals excite the production of antitoxins.

Preventive Inoculations.—Recovery from one attack of symptomatic anthrax protects an animal against a second infection. Active immunity can be produced by vaccines of attenuated organisms. A dried powder of the muscles of animals which have succumbed to the disease is used as a vaccine and subjected to a suitable temperature to insure attenuation of the virulence of the spores contained therein. Two vaccines are prepared—a stronger vaccine by exposing a portion of the powder to a temperature of 85° to 90° C. for six hours, and a weaker vaccine by exposing it for the same time to a temperature of 100° to 104° C. Inoculations are made with this attenuated virus into the end of the tail—first the weaker and later the stronger. The results obtained from this method of preventive inoculation seem to have been very satisfactory.

BACILLUS WELCHII GROUP (BACILLUS (AËROGENES) CAPSULATUS, BACILLUS PERFRINGENS, CLOSTRIDIUM WELCHII).

The first bacillus of this group to be described minutely was found by Welch in the bloodvessels of a patient suffering with aortic aneurysm; at autopsy, made in cool weather, eight hours after death, the vessels were observed to be full of gas bubbles. Since then it has been found in many cases in which gas has developed from within sixty hours of death until some hours after death. It occurs most frequently after external cutting operations and wounds. These cases are, as a rule, marked by delirium, rapid pulse, high temperature, and the development of emphysema and discoloration of the diseased area or of marked abdominal distention when the peritoneal cavity is involved. Members of this group are present, as a rule, in the intestinal canal of man and animals and are apt to be found in the dust of hospitals and elsewhere. Herter[1] has shown that they are present in excessive numbers in certain diseases of the digestive tract. These cases are apt to develop anemia.

Different strains of bacilli belonging to this group have appeared under different names and their exact relationship is still uncertain. Thus B. phlegmonis emphysematosæ of Fränkel is probably the same as B. welchii. B. perfringens (Veillon and Zuber, 1898) and B. enteritidis sporogenes (Klein, 1895) are closely related if not the same organism.[2]

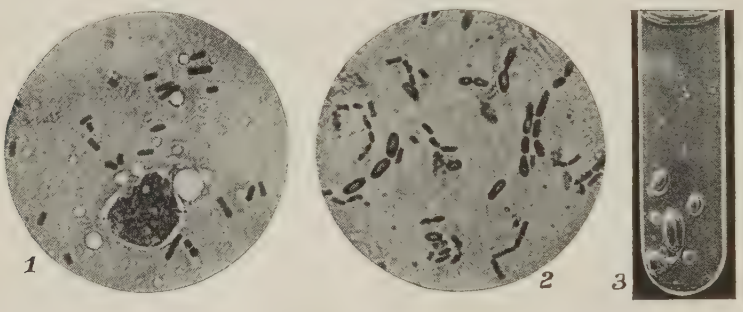


Fig. 159.—Bacillus aërogenes capsulatus. 1, bacilli, 2, spores; 3, culture in dextrose-nutrient agar.

Morphology.—Straight or slightly curved rods, with rounded or sometimes square-cut ends; somewhat thicker than the anthrax bacilli and varying in length; occasionally long threads and chains are seen. The bacilli in the animal body, and sometimes in cultures, are enclosed in a transparent capsule. Spores are usually absent in the tissues and often in cultures. Dunham showed that the culture isolated by Welch formed spores when grown on blood serum. Some strains since isolated make spores readily.

[1] Jour. Biol. Chem., 1906, 2, 1.
[2] Simonds: Jour. Inf. Dis., 1915, 1, 31.

Biology.—Anaërobic non-motile bacilli, positive to Gram, but more easily decolorized than many other Gram-positive bacteria. Growth is rapid at 37° C., in the usual culture media containing certain sugars, and is accompanied by the production of gas. *Nutrient gelatin* is not liquefied. On *agar* colonies are developed, which are from 1 to 2 mm. or more in diameter, grayish-white in color, and in the form of flattened spheres, ovals, or irregular masses, covered with hair-like projections. *Bouillon* is diffusely clouded, and a white sediment is formed. *Milk* becomes rapidly acidified and coagulated, assuming a worm-eaten appearance which has been called stormy fermentation. A large amount of butyric acid is produced. They ferment most sugars with much gas formation. They have been divided by Simons[1] into four groups according to their ability to ferment either inulin, or glycerol, or both or neither, but they apparently all produce the same toxin.

Isolation.—When quantities up to 2.5 c.c. of fresh bouillon cultures are injected into the circulation of rabbits and the animals killed shortly after the injection, the bacilli develop rapidly, with an abundant formation of gas in the bloodvessels and organs, especially the liver. This procedure is a good method of obtaining the bacilli. The material suspected of containing the bacillus alone or associated with other bacteria is injected intravenously into rabbits, which are killed five minutes later and kept at 37° C. for sixteen hours, and cultures in milk made from the liver and heart blood. If stormy fermentation occurs in the milk after incubation, the presence of B. welchii may be assumed. In order to obtain a pure culture anaërobic plate cultures should be made.

In war wounds B. welchii is usually accompanied by *B. sporogenes* (Metchnikoff, 1908) a markedly proteolytic anaërobe which is non-pathogenic in itself, but which probably stimulates the pathogenicity of other anaërobes. The presence of this anaërobe makes the isolation of B. welchii in pure culture often a difficult procedure.

Pathogenicity.—Its pathogenicity is usually not marked in healthy animals, although Dunham found that the bacillus taken freshly from human infection is sometimes very virulent.

As we have said, bacilli of this type are one of the frequent infections after irregular unclean wounds such as those received in war. In these infections there is marked destruction of tissue, especially in the muscles. This has been supposed to be due to one or more of five conditions: (1) to the large quantity of butyric acid produced from the glycogen of the muscles; (2) to pressure from the amount of gas produced; (3) to presence of foreign protein; (4) to specific toxin; (5) to the presence of a soluble, ionizable *calcium* salt which, according to Bullock and Cramer,[2] produces a local breakdown of the normal defensive mechanism against spore-bearing anaërobes. Many attempts have been made to demonstrate a specific toxin production by these bacilli

[1] Rockefeller Institute Monograph No. 5, 1915.
[2] Proc. Roy. Soc., London, 1919, **91**, 513.

(Harde,[1] Weinberg,[2] Bull and Pritchett[3]), and the last three have demonstrated that an exotoxin is produced which is controlled by an antitoxin. Robertson[4] has made a study of the isolation and types of anaërobes found in wounds.

It is suggested by Welch that in some of the cases in which death has been attributed to the entrance of air into the veins the gas found at the autopsy may not have been atmospheric air, but may have been produced by this or some similar microörganism entering the circulation and developing shortly before and after death. The same may be true for gas in the uterine cavity.

B. Fallax (Clostridium Fallax).—Weinberg and Seguin found this form occasionally associated with the other anaërobes in war wounds. It is slightly pathogenic. It coagulates milk slowly. It does not liquefy gelatin or coagulate serum.

B. Histolyticus (Clostridium Histolyticum).—This was also discovered by Weinberg and Seguin in war wounds. It is intensely proteolytic digesting tissue rapidly down to the bone in test animals who seem to show no general reaction.

B. Putrificus (Clostridium putreficum) discovered by Bienstock in 1884, one of the three great putrefying bacteria in Nature, was also found occasionally in war wounds. It is non-pathogenic. (See large table at end of Part I).

BACILLUS ŒDEMATIENS.

This organism was isolated by Weinberg and Seguin as a new species, but a number of workers think it is the same as the anaërobe isolated by Novy in 1894[5] from an edematous infection in guinea-pigs. Novy called his organism B. œdematiens maligni II. This name was changed to Clostridium Novyi. Weinberg and Seguin describe their organism as follows: It is rather a stout bacillus, occurring singly or in long chains. It is Gram-positive and sluggishly motile in ordinary media. It possesses very many flagella. It sporulates in all media, even when glucose is added. The spores are usually subterminal. It is a very strict anaërobe, growing with difficulty. This is the reason why so many workers have missed it.

The colonies in deep, nitrated agar are at first opaque in the center and surrounded by unequal filaments. Later, they become clearer, the center cloudy, with a periphery of hairy filaments (very much like tetanus or Vibrion septique colonies).

In Martin's broth the growth is first slightly cloudy, then the broth becomes perfectly clear, a precipitate settling at the bottom. This bacillus ferments glucose, maltose, lactose, saccharose, glycerin and starch. Acid production is not great, though there is considerable gas

[1] Compt. rend. Soc. de Biol., 1915, **78**, 134.
[2] Proceed. Roy. Soc. Med., 1916, **9**, 119.
[3] Jour. Exp. Med., 1917, **26**, 867.
[4] Jour. Path. and Bact., 1916, **20**, 327.
[5] Ztsch. Hyg., 1894, **17**, 209.

production. It does not liquefy coagulated horse serum. It secretes a very active soluble toxin which stimulates the production of an antitoxin.

Weinberg and Seguin found other strains similar to their own culturally and morphologically, but not at all pathogenic. Their strains are very virulent for the laboratory animals and produce a clear, gelatinous edema.

Bacillus Bellonensis.—A form isolated by Sacquepée (1916) as a new species but thought by some to be the same as B. œdematiens.

SERUM TREATMENT OF "GAS GANGRENE."

Both preventive and curative treatments of the wound condition known as "gas gangrene" were tried during the Great War. Statistics in regard to their practical efficacy were on the whole favorable. In using serum as a curative agent the following four main factors should be considered:

1. The specificity of the serum against the particular infection. Thus the serums of Bull and Pritchett and Veillon produced only against the perfringens (B. welchii) are not potent if the infection is caused by edematiens or vibrion septique.

2. The time of application of serum. It should be injected as early as possible after the receipt of the wound. It is of no value when the infection has reached the stage of septicemia.

3. Thorough surgical procedure: all dead tissue should be removed and the wound thoroughly cleaned, so as to remove the favoradle soil for the anaërobes and encourage phagocytosis by the healthy tissue.

4. Dosage and mode of injection. Enough serum should be given to neutralize the poison generated by the gas gangrene organisms. The injections are preferably intravenously, or may be given also intramuscularly around the wound, and repeated as often as necessary according to the condition of the patient and bacteriological findings.

Preventive serotherapy is by far the most satisfactory and is clearly outlined by some of the French surgeons (Delbet, Duval, Vaugher) in their recommendations. The British and Americans adopted this procedure even before the French.

Favorable results of the prevnetive treatment have been reported by the French, English and American surgeons. Thus Duval and Vaugher give results on 381 cases which received preventive treatment. Only 4.7 per cent. developed gas gangrene as compared with from 15 per cent. to 18 per cent. of those (same category of cases) which were not subjected to preventive treatment.

Van Beuren[1] has published a summary of the subject to that date.

Nevin[2] showed experimentally that mixed serums for the different types of pathogenic anaërobes were very effective against mixed infection, and as this mixed infection nearly always occurred, she recommended that such mixed serums should be given without waiting to determine the type of the organism.

[1] Jour. Am. Med. Assn., 1919, **73**, 239.
[2] Jour. Inf. Dis., 1919, **25**, 178.

CHAPTER XXXIV.

THE CHOLERA SPIRILLUM (VIBRIO COMMA) AND SIMILAR VARIETIES.

In 1883 Koch separated a characteristically curved organism from the dejecta and intestines of cholera patients—the so-called *"comma bacillus."* It was absent from the blood and viscera, and was found only in the intestines; and the greater the number, it was said, the more acute the attack. Koch also demonstrated an invasion of the mucosa and its glands. The organisms were found in the stools on staining the mucous flakes or the fluid with methylene blue or fuchsin, and sometimes alone; by means of cultivation on gelatin they were readily isolated from the stools. Numerous control observations made upon other diarrheic dejecta and upon normal stools were negative; the comma bacillus was found in choleraic material only, or occasionally in small numbers in the stools of healthy persons who came in contact with cholera. Soon, however, other observers described comma-shaped organisms of non-choleraic origin. Finkler and Prior, for instance, found them in the diarrheal stools of cholera nostras, Deneke in cheese, Lewis and Miller in saliva. All of these organisms, however, differed in some respects from Koch's comma bacillus, *and it has since been proved that none of them is affected by the specific serum of animals immunized to the cholera vibrio.* After a time, therefore, the exclusive association of Koch's vibrio with cholera or those in contact with it became almost generally acknowledged, until now it is regarded by bacteriologists everywhere to be the specific cause of Asiatic cholera. Certain sporadic cases of cholera-like disease, however, are undoubtedly due to other organisms.

Morphology.—Curved rods with rounded ends which do not lie in the same plane, of an average of $1\frac{1}{2}\mu$ in length and about 0.4μ in breadth. The curvature of the rods may be very slight, like that of a comma, or distinctly marked, particularly in fresh unstained preparations where the adhesion of two individuals presents the appearance of a half-circle. By the inverse junction of two vibrios S-shaped forms are produced. Longer forms are rarely seen in the intestinal discharges or from the cultures grown on solid media, but in fluids, especially when grown under unfavorable conditions, long, spiral filaments may develop. The spiral forms are best studied in the hanging drop, for in the dried and stained preparations the spiral character of the long filaments is often obliterated. In film preparations from the intestinal contents in typical cases it will be found that the organisms are present in enormous numbers, and often in almost pure culture. In old cultures

irregularly clubbed and thickened involution forms are frequent, and the presence in the organisms of small, rounded, highly refractile bodies is often noted.

Staining.—The cholera spirillum *stains* with the anilin colors usually employed, but not as readily as many other bacteria; a diluted aqueous solution of carbol-fuchsin (1 to 10) is the most reliable staining agent. The vibrion is decolorized by Gram's method. The organisms exhibit one long, fine flagellum attached to one end (other spirilla often have two or more end flagella).

Biology.—The cholera spirillum is aërobic, liquefying, and extremely motile. It grows readily on ordinary culture media, best at 37° C., but also at room temperature, 22° C.

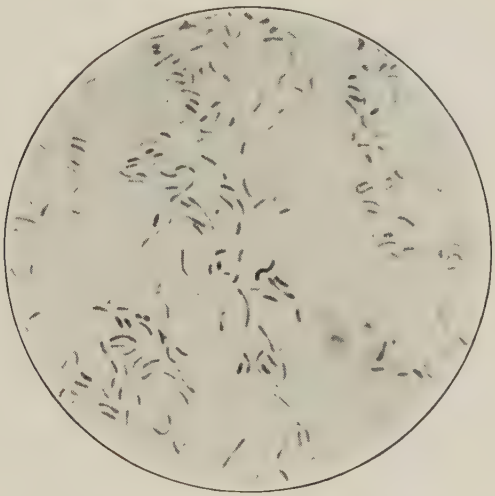


FIG. 160.—Spread of pure cultures of cholera spirillum from a twenty-four-hour growth on nutrient agar. × 1700. Stained with fuchsin. (Bailey and Siegel.)

Gelatin Plate Cultures.—In gelatin plate cultures, characteristic colonies are produced. After twenty-four hours' growth, there is a uniform granular appearance of the surface, which because of the high degree of refraction gives the appearance of being powdered with broken glass. Color is practically absent or there may be a slight yellowish tint thus differing from B. coli. If growth is continued, liquefaction appears about the colony and its appearance gradually changes. The characteristic colony in gelatin used to be of the greatest practical importance. With the introduction of special selective media, however, the use of gelatin has been practically discontinued.

Gelatin Stab Cultures.—In gelatin stab cultures, a small funnel of liquefaction appears after twenty-four hours. This deepens and broadens, until at the end of a week liquefaction may be complete.

Agar.—On agar a moist, shiny, grayish-yellow layer develops. On the surface of alkaline-agar plates the individual colonies are characteristic.

They are round, transparent and have a rather distinctive opalescent sheen. This characteristic appearance is made use of for isolation.

On **Dieudonné alkaline-blood agar** the colonies are large and circular. By transmitted light there is a glassy transparency, by reflected light they are grayish. On the **alkaline-egg agar** (Krumwiede) the colonies, when examined by transmitted light, appear to be deep in the agar and have a distinctive hazy appearance due to the development of a halo about the colony. A zone of clearing may develop where growth is continued. As some fecal bacteria other than vibrios may develop on these media, the development of a typical colony is a great aid in isolation.

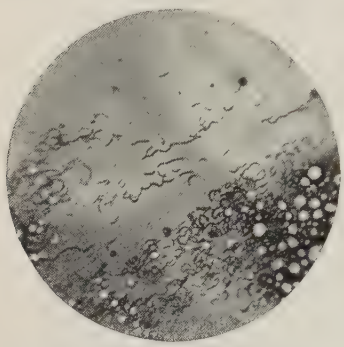


FIG. 161.—Cholera spirilla preparation from gelatin-plate culture of cholera. × 800 diameters.

Löffler's blood serum is rapidly liquefied at 37° C. **Milk** is not coagulated. **Glucose** and **saccharose** are fermented with great rapidity, acid only being formed. Growth in fluid media is abundant and usually characteristic, most so in peptone-water. **Peptone-water** is diffusely cloudy to a moderate degree, but at the surface the cloud is much denser. Due to the greater supply of oxygen, the vibrios seek the surface and multiply there more freely.

Reaction of Media.—The cholera vibrio grows best on media that are strongly alkaline to litmus. They can grow in an excess of alkali sufficient to inhibit the growth of many of the bacteria found in feces. This is most evident where an alkali-albumin mixture is used to increase the alkalinity. (See Special Media.)

Cholera-red Reaction.—All cholera strains give this reaction. (Described in Chapter IV.) This is important as many non-cholera spirilla do not give it.

Changes due to Artificial Cultivation.—Cholera strains which have been in cultivation for some time show more spiral forms. They grow less typically or not at all on selective media and fluid cultures may develop a wrinkled pellicle. Their digestive powers are also lessened, as gelatin-liquefaction or the liquefaction of Löffler's serum medium. Variations can also be induced by special conditions.

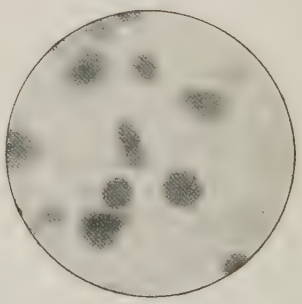


FIG. 162.—Cholera colonies in gelatin; twenty-four to thirty-six hours' growth. × about 20 diameters.

Hemolysin Production.—Six strains were isolated by Gottschlich at El Tor from Pilgrims to Mecca, who died with diarrheal symptoms but had shown no clinical evidences of cholera. These strains are called "El Tor" strains. They give the serum reactions of cholera vibrios

but produce a strong hemolysin. Kraus and Ruffer believed them not to be true cholera, as hemolysin production was considered by the former as an attribute possessed only by non-cholera types. Further investigations have shown that cholera vibrios may develop, lose, or show wide variations in their ability to produce hemolysins. It is apparently a potential power of all vibrios and of no absolute value in differentiation.

Resistance and Vitality.—Development Outside of Body.—If a culture is spread on a cover-glass and exposed to the action of the air at room temperature the spirilla will be dead at the end of two or three hours, unless the layer of culture is very thick, in which case it may take twenty-four hours or more to kill all the spirilla. This indicates that infection is rarely if ever produced by means of dust or other dried objects contaminated with cholera spirilla. The transmission of these organisms through the air, therefore, can only take place for short distances, as by the spray from infectious liquids.

The cholera vibrio is also injuriously affected by the abundant growth of saprophytic bacteria. It is true that when associated with other bacteria, if present in large numbers, and if the conditions for their development are particularly favorable, the vibrio may at first gain the upper hand, as in the moist linen of cholera patients, or in soil impregnated with cholera dejecta; but later, after two or three days, even in such cases, the vibrios die off and other bacteria gradually take their place. Thus, Koch found that the fluid contents of privies twenty-four hours after the introduction of cholera vibrios no longer contained the living organisms; in impure river water they were not demonstrable for more than six or seven days, as a rule. In the dejecta of cholera patients they were found usually only for a few days (one to three days), though rarely they have been observed for twenty to thirty days, and on one occasion for one hundred and twenty days. In unsterilized water they may also retain their vitality for a relatively long time; thus, in stagnant well water they have been found for eighteen days, and in an aquarium containing plants and fishes, the water of which was inoculated with cholera germs, they were isolated several months later from the mud at the bottom. Koch found them in the foul water of a tank in India, used by the natives for drinking purposes. In running river water, however, they have not been observed for over six to eight days. In milk they are finally destroyed by acidity due to the growth of the milk bacteria, in sterilized milk they may survive eight to ten days. For the cholera organisms the conditions favorable to growth are a warm temperature, moisture, a good supply of oxygen, and a considerable proportion of organic material. These conditions are fully met, outside the body, in very few localities.

The cholera vibrio has the average resistance of spore-free bacteria, and is killed by exposure to moist heat at 56° C. in half an hour, at 80° C. in five minutes, at 95° to 100° C. in one minute. The vibrios have been found alive after kept a few days in ice, but ice which has been preserved for several weeks does not contain living organisms.

Chemical disinfectants readily destroy the vitality of cholera vibrios. For disinfection on a small scale, as for washing the hands when contaminated with cholera infection, a 0.1 per cent. solution of bichloride of mercury, or a 2 to 3 per cent. solution of carbolic acid or, better, lysol may be used. For disinfection on a large scale, as for the disinfection of cholera stools, strongly alkaline milk of lime is an excellent agent. The wash of cholera patients, contaminated furniture, floors, etc., may be disinfected by a solution of 5 per cent. carbolic acid and soap water. For the disinfection of drinking water, chlorinated lime, 1 or 2 parts per million of available chlorine may be used for fairly pure water. Five parts per million would probably be effective even in polluted water.

Pathogenesis.—Not one of the lower animals is naturally subject to cholera. Koch succeeded in producing symptoms and intestinal lesions in guinea-pigs similar to those in man by introducing cultures by catheter after neutralizing the contents of the stomach with a solution of carbonate of soda and inhibiting peristalsis by the use of opium. Thomas injected a dilute suspension of cholera spirilla into the ear vein of young rabbits, and thus caused symptoms and lesions of the intestinal mucous membrane resembling those of cholera in man. The spirillum was recovered from the feces. Metchnikoff was successful with suckling rabbits by rubbing a small amount of a culture on the teats of a mother rabbit. Intraperitoneal injections with cholera spirilla kill guinea-pigs acutely, but intestinal lesions are rarely found.

Accidental Human Infection.—There are several cases on record which furnish the most satisfactory evidence that the cholera spirillum is able to produce the disease in man. In 1884 a student in Koch's laboratory in Berlin, who was taking a course on cholera, became ill with a severe attack of cholera. At that time there was no cholera in Germany, and the infection could not have been produced in any other way than through the cholera cultures which were being used for the instruction of students. In 1892 Pettenkofer and Emmerich experimented on themselves by swallowing small quantities of fresh cholera cultures obtained from Hamburg. Pettenkofer was affected with a mild attack of cholerine or severe diarrhea, from which he recovered in a few days without any serious effects, but Emmerich became very ill. On the night following the infection he was attacked by frequent evacuations of the characteristic rice-water type, cramps, tympanites, and great prostration. His voice became hoarse, and the secretion of urine was somewhat diminished; this condition lasting for several days. In both cases the cholera spirillum was obtained in pure culture from the dejecta. Finally, there is the case of Dr. Oergel, of Hamburg, who accidentally, while experimenting on a guinea-pig, allowed some of the infected peritoneal fluid to squirt into his mouth. He was taken ill and died a few days afterward of typical cholera, though at the time of his death there was no cholera in the city.

Distribution in the Body.—The cholera spirilla are found mainly in the intestines and it is believed that any invasion of the blood stream

is slight. A demonstrable bacteriemia is absent. The lower half of the small intestine is most affected, a large part of its surface epithelium becoming shed. The flakes floating in the rice-water discharges consist mostly of masses of epithelial cells and mucus, among which are numerous spirilla. The spirilla also penetrate the follicles of Lieberkühn, and may be seen lying between the basement membrane and the epithelial lining, which become loosened by their action. They are rarely found in the connective tissue beneath, and never penetrate deeply. In more chronic cases other microörganisms play a greater part and deeper lesions of the intestines may occur. They are not infrequently found in the gall-bladder and may cause a cholecystitis.

Cholera Toxins.—Koch assumed that the severe symptoms of cholera were due to the absorption of a toxin produced by the growth of the vibrio in the intestines. The toxic effects are apparently due to substances which are an integral part of the organism and are only liberated by the breaking down of the vibrio. Suspensions of killed vibrios, when injected into animals, give the same symptoms as living cultures, although quantitatively less toxic. The endotoxin is labile, and is best shown in cultures which are killed by chloroform or by heating to 56° C. for one hour. More active chemicals or a higher degree of heat changes it from a more specific toxin to a more general protein poison. The bacteria-free filtrates of fresh fluid cultures are only slightly toxic; old cultures, however, due to breaking down of the vibrios may be very toxic. This toxicity is due mostly to substances similar in action to the general class of ptomaines. Kraus has, however, been able to demonstrate what he considers an extracellular toxin in young broth cultures. Metchnikoff and Roux have also attempted to prove the existence of an extracellular toxin by growing cholera vibrios in collodion sacs implanted in the peritoneum of guinea-pigs. The production of an antitoxin against such toxins has not been accomplished.

Communicability.—Origin of Epidemics.—The two fundamental epidemiological facts are, that the vibrio leaves the body only in the feces, and the mode of infection is by way of the mouth. Vibrios are probably excreted in small numbers during the incubation period. The feces of the *cholera patient* during the acute stage of the disease are extremely rich in vibrios, which are at times present in almost pure culture. As the case recovers they decrease in number, but persist after recovery in most cases for only seven to fourteen days. In isolated instances they may be demonstrable in the intestinal discharges for three months. In some cases the vibrios may still be present in the gall-bladder when not demonstrable in the feces. It is believed that such cases may excrete cholera vibrios intermittently as long as an inflammatory condition persists in the gall-bladder. These persons constitute the "*convalescent carriers.*" In this connection, the mild cases which are undiagnosed or overlooked are important. Another group of persons may act as sources of infection, viz., excrete cholera spirilla in their stools. These are the "*healthy* or *contact carriers.*" Not all persons who ingest cholera develop the disease. In a number, the vibrio will

multiply in the intestine to a limited extent and be excreted in the stools, although no clinical evidences of disease are present. These healthy carriers are important not only as insidious spreaders of infection, but they may be potential cases of cholera. Should their resistance be lowered they may develop the disease.

The transfer of the infectious agent to the mouth may occur in several ways: by personal contact, by fomites, and by contamination of food and water. Where a moderate number of cases are developing in a district having fair sanitary conditions, contact, especially with mild cases and carriers, or indirectly, fomites or infection of food are the sources of infection. As other factors enter, such as the contamination of the soil and privy-vaults with subsequent infection of well and river water and of green vegetables, the cases increase in number. Where a general water supply becomes contaminated an explosive, widespread epidemic follows. It is easy to understand how localized epidemics are kept active, by the development of carriers, who help maintain the contamination of their surroundings; and, given also the climatic conditions, such as heat and moisture, to favor the continued vitality or even multiplication outside of the body, how endemic foci persist. The transfer of infection by fomites, such as body and bed linen or dishes, etc., is only dangerous when direct. Drying quickly lessens the danger. Flies may be a factor by mechanical transfer of the virus.

The susceptibility to infection of different individuals varies and conditions may lower or raise the resistance of the individual. The occurrence of healthy carriers illustrates this. Such carriers may be very numerous. Abel and Clausen, for example, found that 14 of 17 persons belonging to families of 7 cholera patients, had cholera vibrios in their stools. In a group of immigrants who were exposed, we found 10 per cent. were healthy carriers.

The resistance of the individual depends upon his general good health. Gastric and intestinal disorders due to indiscreet eating or drinking, or other causes undoubtedly favor infection or in the case of healthy carriers may cause the development of cholera. At the New York Quarantine Station two such cases promptly developed: one after the administration of a dose of salts, the other after a drinking bout.

Cholera Immunity.—Eight to ten days after recovery the serum of cholera patients contains protective substances. If a guinea-pig is injected intraperitoneally with living cholera vibrios, and serum from the patient be given, the pig recovers. Similar protective substances are found in the serum of animals injected with sublethal doses of live vibrios or with killed organisms. The serum is not antitoxic, for although it will protect an animal from a lethal dose of living vibrios, by preventing their multiplication, it has little effect, when a fatal dose of killed organisms or toxic extracts is given. When vaccinated *per os* by Besredka's method animals may be refractory to intestinal inoculation.[1]

[1] Horowitz, Wlassowa and Pirojnikowa: Am. Inst. Past., 1926, **40**, 727.

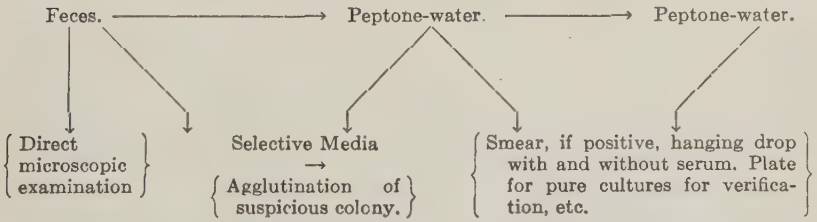
The antibodies present in the serum are precipitins, agglutinins, opsonins and bacteriolytic substances.

Prophylactic Vaccination.—Prophylactic vaccination is stated to afford considerable protection, but the mortality-rate of those who become infected is only slightly influenced. The reactions may be moderately severe.

Specific Serum Reactions.—Agglutinins.—Because of the acuteness of the disease, the agglutination reaction is valueless for diagnosis. It may be of diagnostic help in recovered cases, where no bacteriological diagnosis has been made. Agglutination is used, however, for identification of the cholera vibrio. In this way cholera and non-cholera vibrios can be separated with certainty, because a cholera-immune serum usually contains few group agglutinins for non-cholera types. Freshly isolated strains agglutinate freely; in fact, seem to give better reactions than stock cultures, although strains vary in their agglutinability; relatively inagglutinable strains such as are encountered among freshly isolated strains of typhoid apparently have not been found. Reports indicate that vibrios differing from the typical cholera vibrio in agglutinative reactions are encountered in certain areas in choleraic disease. These vibrios have been termed paracholera. The choleraic disease with which they have been associated has been relatively mild, sporadic, or if in outbreaks, in those of limited extent.[1]

Pfeiffer Phenomenon.—This phenomenon (see Chapter XI), which is a specific bactericidal test *in vivo*, was employed by early workers to differentiate the cholera vibrio from other vibrios. The bactericidal serum should have a high titer, viz., 1 c.c. of a 1 to 1000 dilution should be able to dissolve a loop of cholera vibrios when injected intraperitoneally together. Each factor in the test must be controlled. The agglutination method is a more simple means of identification.

ISOLATION OF CHOLERA VIBRIO FROM STOOLS. OUTLINE OF PROCEDURE.[2]



Direct Microscopic Examination.—This is of great value in suspected cases but of no use in the examination of carriers. The presence of many typical, extremely motile vibrios warrants a tentative diagnosis

[1] Douglas: British Jour. Exp. Path., 1921, **2**, 49; Mackie: British Jour. Exp. Path., 1922, **3**, 231.

[2] Krumwiede, Pratt and Grund: Jour. Inf. Dis., 1912, **10**, 134.

of cholera. In exceptional cases, not cholera, a great abundance of vibrios may be found. This led us in one instance unnecessarily to isolate a nurse, who while caring for a cholera patient, developed nervous diarrhea.

Peptone-water.—Inoculate with feces and incubate six to twelve hours. Examine smears from surface growth. If the vibrios are numerous, prepare hanging drops with and without immune serum. If vibrios are not found in the smear, or if too few in number for hanging drop observation, subinoculate into peptone-water, or selective media, or both.

Subculture Peptone-water.—This second enrichment is probably never required except in the examination of suspected carriers. In four instances we have found cholera vibrios in subculture where they were not evident in smears from the first peptone culture. This second enrichment helps also to exclude some of the vibrios other than cholera; some will have died out, some will not have enriched beyond the amount present in the first peptone culture; these are not cholera. Smears from the surface growth are examined and if positive, examination of hanging drops carried on. Where haste is not a factor as in carrier examinations the examination of the first peptone culture may be omitted.

In making smears and hanging drops from peptone-water especial care should be taken not to shake the tubes and that the loop is just touched to the surface. In the examination of smears little time need be spent; if very few vibrios are present, further enrichment is necessary. If they are present in sufficient numbers for agglutination, they are found at once.

The use of the surface growth for agglutination is open to certain criticisms, but in practical work it has given us accurate results. Where cholera or cholera-like vibrios, having the same ability to enrich as true cholera, are present, the surface growth is nearly a pure culture. The reliability of the peptone method, as outlined, was best shown in a series of examinations where we found two carriers. In this series 50 per cent. of the stools contained vibrios. The results were checked by the isolation of pure cultures. With some experience a great number of examinations can be carried through in this way, using peptone-water only, with a minimum of preparation and equipment.

Selective Media.—Inoculation may be done directly from the feces or after enrichment in peptone-water. The advantage of such media is that they may be heavily inoculated. The colonies which develop are used for agglutination either in hanging drop, or more convenient, the macroscopic slide method (see under Typhoid). Various modifications of Dieudonné's have been suggested. The alkaline-egg medium has the advantage that a distinctive vibrio colony develops allowing quick selection for agglutination should non-vibrios develop. A secondary plating from selective media to plain agar is necessary, if pure cultures are to be obtained. The other bacteria, which are only restrained, may be transferred in fishing a colony and thus yield mixed cultures.

Alkaline agar may be employed for plating either directly from the

feces or from peptone enrichments. As stated, the colony is distinctive and no difficulty will be encountered if the vibrios are fairly numerous.[1]

Saccharose Peptone-water.—This medium has been suggested by Bendick to avoid the time-consuming microscopic examination of peptone tubes. The stool is first inoculated in peptone-water and the surface growth then inoculated into this medium. Because of the ability of the cholera vibrio to rapidly split saccharose, decolorization of the phenolphthalein occurs in five to eight hours. The tubes which do not decolorize in this time can be discarded, those decolorized are examined for vibrios, which, if present, are isolated by plating.

Because of the presence of a fermentable sugar, the growth is diffused and the surface is not satisfactory for agglutination. To avoid this difficulty duplicate peptone tubes could be planted, however, and used for agglutination when the saccharose peptone tubes were decolorized. This method should be of the greatest value where many specimens must be examined in the search for carriers.

Examination of Suspected Carriers.—The simplest procedures possible must be employed when the daily examinations may run into many hundreds or even thousands. The peptone method outlined reduces the bacteriological work very much, especially if the first peptone tubes are not examined. The collection of stools under these circumstances is impracticable. Ordinary swabs moistened in peptone-water may be inserted into the rectum to obtain fecal material. Individual glass specula may be employed to aid in the introduction of the swabs. Where feasible much time is saved by giving an identification card a number and dropping the swab, at the time of taking, into a similarly numbered tube of peptone-water. The sterilization of the peptone tubes, supported in blocks of wood or racks protected by a cover instead of by cotton plugs, and the use of individual wire loops (sterilized in bundles) for transfers or smears saves a great deal of time. The removal and replacing of cotton plugs and the burning of the platinum loop usually employed is thus avoided. In transferring, the wire is dropped into the second tube. The exposure of the tubes when the cover is removed for inoculation or transfer does not lead to interference by contamination.[2]

Isolation from Water.—As the cholera vibrios are few in number in water a large volume should be used. About 1 liter of water is taken and 100 c.c. amounts placed in Ehrlenmeyer flasks and to each is added 10 c.c. of a tenfold strength peptone-water. These are shaken and incubated for eighteen hours. The surface growth is then subinoculated in peptone-water for further enrichment if necessary and plates made for isolation. Especial care must be taken in fishing as vibrios other than cholera may be present in the water, and there is some evidence that cholera vibrios lose their agglutinability to some extent after living in water. These difficulties may be surmounted by many fishings and the use of a high titre serum in low dilutions in order to select cultures for final identification.

[1] The direct plating of stools on agar or gelatin is sometimes of practical importance. In no disease but cholera do vibrio colonies approach or exceed in number those developing from the ordinary fecal bacteria. Even without identification by agglutination, such a condition gives us a practically certain diagnosis of cholera.

[2] Creel: Am. Jour. Public Health, December, 1911.

Spirilla (Vibrios) More or Less Allied to the Cholera Spirillum.—Various types of spirilla may be isolated from stools, water, and other sources. Some are practically identical, morphologically and culturally, with true cholera. It would be well to limit the term "cholera-like vibrios" to this group. Another group of vibrios, similar to cholera in some respects but differing in others might be termed "non-cholera vibrios." Much of the practical interest in these types was lost when the serological methods for identification were introduced. In emergency work, where immune serum is not at hand, they are of extreme practical importance. A few of the types are of interest because of their pathogenicity for animals.

Some of these vibrios enrich in mixed cultures in peptone-water like true cholera, others enrich to a limited extent or die out. The majority of the vibrios found in stools during routine examinations for cholera spirilla can be excluded culturally if serum be not available. Of 50 vibrios thus isolated we found that 43 did not give the cholera-red reaction. Of the 7 that did, 2 produced no acid from glucose and 4 produced acid and gas. One produced acid only, but could be excluded, as it formed a tenacious pellicle on peptone-water.

The following is a short list of vibrios of interest historically or because of their pathogenicity for animals:

Vibrio metschnikovii; source, epizoötic gastro-enteritis of fowls, present in intestinal contents and in blood. Typical vibrio with one flagellum, liquefies gelatin and gives cholera-red reaction. A minute amount of culture inoculated into a cutaneous wound causes a fatal vibriosepticemia in pigeons and guinea-pigs.

Vibrio massavah; source, stools, considered cholera vibrio when first isolated, four flagella, pathogenicity like Vibrio metschnikovii.

Vibrio septicus; source, stools, case of cholerine, cholera-like culturally and morphologically, minute amounts cause a rapidly fatal septicemia in guinea-pigs.

Spirillum finkler-prior; source, feces in cholera nostras, does not give cholera-red reaction.

Vibrio ivanoff and *Vibrio berlionensis;* source, former artificially inoculated stools for disinfection tests, latter, water artificially inoculated to determine viability of cholera vibrio in water. Both are probably variants produced by artificial conditions; they give the immune reactions of cholera spirilla.

CHAPTER XXXV.

SPIROCHETA AND ALLIES.

The genus *Spirocheta* (coiled hair) was introduced in 1838 by Ehrenberg, who differentiated it from spirillum by its flexibility. Schaudinn, in 1905, thought he saw an undulating membrane in *Spirocheta refringens* so he added this characteristic to the genus and considered that thus its relationship to the flagellated protozoa, genus *Trypanosoma*, was indicated.

Numerous spirochetes and spiral organisms have been described, some associated with *Treponema pallidum* in syphilis, some in other lesions or in the normal secretions of both man and the lower animals; and still the question as to their classification is unsettled.

The chief reasons given for considering spirochetes protozoa are: The flexibility and the indications in many of longitudinal division and of undulating membrane: the demonstration of forms intermediate between the trypanosomes and the spirochetes; the spirochetal forms of certain trypanosomes (T. noctuæ); stages of development in the louse and tick; dissolution by certain chemicals such as saponin or sodium taurocholate in contrast to the resistance shown by most bacteria.

In favor of the bacterial nature of spirochetes are: The rigidity of some forms, the lack of undulating membrane in most and of definite nuclear apparatus in all, and the evidence of transverse division in all and of flagella arising from the periplast in some.

The chief differences between the six genera now accepted are tabulated[1] on page 574.

It may be well to note briefly the chief characteristics of the more familiar non-pathogenic species in order better to understand the relationships between them and the *Treponema pallidum* and other pathogenic forms.

Material and Methods for Study.—A large spiral organism (Cristispira balbianii) is found in the stomach of oysters fresh from salt water. Smaller spirochetes are frequently found in human mouths, in intestines, in water, slime and so on. When fresh syphilitic or relapsing fever material can be obtained this should be examined. The Treponema pallidum (the spiral organism of syphilis), because of its low refractive index, is seen when alive with difficulty by the ordinary microscope, but with the dark-stage illumination, especially if a drop of distilled water is added to the serum containing the organisms, it is seen distinctly and its motion and structure may be more easily studied. The fluid containing the organisms should be dropped on an ordinary glass slide, covered with a thin cover-glass, and well sealed with vaselin, as most spirochetes are anaërobic. Material may be obtained from syphilitic lesions as follows: The

[1] See Bergey's Determinative Bacteriology, 1925, also Noguchi's tabulation in his last review of Spirochetes. Newer Knowledge, Ed. by Jordan and Falk, Chicago Univ. Press, 1928, pp. 452–496.

CHIEF COMPARATIVE CHARACTERISTICS OF SPIRAL MICROBES.

Type of species.	Spirocheta (coiled hair).	Saprospira (putrid coil).	Cristispira (crested coil).	Treponema (turned thread).	Borrelia.	Leptospira (fine coil).
Length	100–500 μ	100–120 μ	30–100 μ	6–14 μ	8–16 μ	7–14 μ
Number of spirals	50–250	100–120	30–100	20–40	10–20	10–20
Number of waves	2–3	3–5	2–5	1–2	3–5	1–with hooked ends.
Axial filament	+	−	−	+[1]	+[1]	+[1]
Terminal filament	−	−	−	+	+	?
Crista	−	−	+	−	−	−
Giemsa stain	Violet	Violet	Violet	Red	Violet	Violet
Triptic digestion	Except axial filament	...	Except cell membrane	Resists many days	Finally digested	Completely digested
Dissolved bile salts, 10 per cent	...	...	Crista +, body −	Slowly	Same	Easily
Saponin, 10 per cent	...	...	Crista +, body −	Immobilized in ½ hr. Broken up in few hrs.	Same	
Plasmolyzed by distilled water	+	...	+	+	+	No effect for many days
Motility	Slow wave creeping	...	Violent rapid lashing and change of place	Rotating rhythmic indulations	Same	Rapid rotations and change of direction. Hooked ends
Habitat	Free living in fresh or salt water	Free living in foraminiferous sand	Parasites in alimentary canal of shell fish	Parasites and pathogens in man and animals cause of syphilis	Same, cause of recurrent fever	Same cause of hemorrhagic jaundice

[1] Whole body an axial filament.

lesion is first thoroughly washed and dried with distilled water and sterile gauze. Part of the base and margin is then scraped with a curette until the superficial tissue is removed and blood appears. The blood is wiped away with sterile gauze until clear serum begins to ooze. A drop of this serum is used for examination.

Smears should be made as thin as possible and may be stained by the method recommended by Giemsa (Tr. pallidum stains reddish) or any modification of the Nocht-Romanevsky methods (see Stains). A modification used by Schereschewsky[1] has been highly recommended by various workers.

Cultures.—Pure cultures are obtained of the Treponema dentium in the following manner: Poured serum-agar plates are made of various dilutions of material from the mouth containing these spirochetes. After being kept in the thermostat at 37° C. under anaërobic conditions for nine to twelve days the spirochetal colonies are fished and planted in agar tubes as stick cultures.

Pure cultures of Borrelia recurrentis (Spirocheta obermeieri) by Novy and of Borrelia refringens by Levaditi have been obtained by growing in collodion sacs.

Noguchi and others have cultivated spirochetes by the same method used for growing Tr. pallidum (see below) and by other methods.

Cristispira Balbiani Certes.—This immense form, next largest known to the Spirocheta plicatilis Ehrenberg, may be found in the oysters' crystalline style. It is important for study because it is apparently a transitional form. In fact it is considered a trypanosome by Perrin[2] and others. Mühlens[3] gives its characteristics as follows: Length 30μ to 120μ, width $\frac{1}{2}\mu$ to 3μ. The body is flattened and possesses a ridge, or crysta, which is visible during life on some individuals. It has 4 to 8 flat, wide spiral coils. Its movements are lively, similar to those of trypanosomes, but more corkscrew-like. During motion its form is apparently easily changed. The rim of the membrane-like ridge does not end in a free flagellum, but one end of it seems to be attached to a triangular mass of chromatin (basal granule, blepharoplast?) which is a part of the central chromatin material. The nuclear material is arranged in a more or less spiral band along the entire center of the organism.

Before division this nuclear band, after passing through chromosome-like changes, breaks up into pairs, and division takes place longitudinally between them. Division is often incomplete for a time, the two ends remaining attached.

The Mouth Spirochetes.—Several species of non-pathogenic forms are commonly found in normal mouths.

Borrelia Buccale, Cohn.—Length, 10 μ to 20 μ; thickness, $\frac{1}{3}\mu$ to $\frac{2}{3}\mu$. It has 3 to 10 irregular flat coils. No true cilia have been demonstrated. The claim of Schaudinn, Hoffmann and Prowazek that it has an undulating membrane has not been corroborated. It stains violet with Giemsa.

Treponema Dentium, Koch.—This is one of the smaller spirochetes. It is as thin as the pallidum and is somewhat similar to it in refraction, staining qualities, and in the fixity of its coils during motion. It is somewhat smaller than the pallidum and stains a little more easily with Löffler's flagella stain, and flagella have been demonstrated. Neither definite undulating membrane nor nuclear material has been seen. It is 4 μ to 12 μ long, and has 4 to 20 regular spirals of about the same appearance as those of the pallidum. Pure cultures have been made from this spirochete as described above. Several varieties have been described.

Treponema macrodentium, a form in appearance between these two, has been found in the mouth. This also is somewhat similar to the pallidum, but it is larger and has less regular spirals; moreover, it stains more intensely with the blue of Giemsa, only in poorly prepared specimens does it appear red.

Treponema microdentium and Treponema mucosum, cultivated by Noguchi

[1] Centralbl. f. Bakt., 1908, **45**, 91.
[2] Arch. f. Protiat., 1906, **7**, 131.
[3] Ztschr. f. Hyg., 1907, **7**, 405; Klin. Jahrb., 1910, 339; Kolle and Wassermann, 2d ed.

in 1912, were distinguished according to him by the ability of the mucosum to produce mucus in cultures. Their size is intermediate between dentium and macrodentium. Mucosum may have some pathogenicity. Leptospira dentate has also been described as occurring in the mouth.

Borrelia refringens.—Borrelia refringens is also found in the mouth, but it is especially interesting because of the fact that it is so often found associated with the Treponema pallidum in the various lesions of syphilis. It is not in such large numbers as the pallidum and probably bears the relation of a restricted secondary invader. It is generally longer than the pallidum (10μ to 30μ) and much thicker ($\frac{1}{2}\mu$ to $\frac{3}{4}\mu$). In life it is much more refractive. It has 3 to 15 irregular wide, flat spirals which change their shape during motion. Its movements are much more lively than those of pallidum. With Giemsa it stains quickly and easily, a blue to a blue-violet tone, according to the length of staining. Levaditi claims to have demonstrated terminal cilia for this organism and to have cultivated it in collodion sacs in the rabbit's peritoneum.

Borrelia vincenti.—Accompanying the fusiform bacilli in Vincent's angina are many spirochetes similar to the "middle form" (Tr. Macrodentium) found in the mouth. Whether they are identical with these spirochetes or whether they are a special variety still remains to be determined. In the meantime Vincent's is listed as a species. Their relationship to the disease is also uncertain.

Borrelia phagedenis.—A rather thick short spirochete, isolated by Noguchi (1913) from phagedenous ulceration on human genitals. It causes acute inflammation in rabbits and rhesus monkeys.

Spirochetes in Tumors.—Löwenthal, Borrel, and others found spirochetes in small numbers in certain mouse tumors. Ewing and Beebe[1] found a few in some dog tumors and others have reported their occasional presence in both ulcerating and non-ulcerating human tumors, but apparently never in sufficient numbers to account for the tissue reaction. Gaylord, however, found that in repeated transplants of a mouse tumor, as the inoculated material became more virulent the number of spirochetes greatly increased. Calkins[2] studied the morphology of Gaylord's spirochete and decided it to be a distinct species. It closely resembles the spirochetes found comparatively frequently by Tyzzer and others in apparently normal mice, though the possibility of infection in these cases was not ruled out.

Miscellaneous Spirochetes.—Besides the spirochetes found in syphilis, in frambœsia, the spiral organisms causing African and European relapsing fevers and those found in infectious jaundice, in rat-bite fever, and in yellow fever, all of which will be described below, spirochetes have been found: (1) In the normal intestinal tract of mosquitoes and human beings as well as in the diarrheal stools of the latter; (2) in the blood of mice and fowls (Bor. gallinarum causing relapsing fever in fowls and Bor. anserina found in similar condition of geese); (3) in various ulcerative and gangrenous processes of man; in certain subacute and chronic pulmonary lesions;[3] (4) in dengue fever.

The fowl spirochetes have been most studied. Marchoux and Salimbeni were the first to show that the tick, Argas persicus, is a carrier of these spirochetes. The mechanism whereby the tick infects the fowl was minutely worked out by Nuttall[4] and his associates.

King and Baeslack report finding a spirochete (S. hyos) in the blood of pigs suffering from hog cholera, which they consider the cause of the disease.

TREPONEMA PALLIDUM (SPIROCHETA PALLIDA).

This organism is found in large numbers in *syphilis*, an infectious disease of human beings, characterized by its long course and by the definite stages of its clinical manifestations.

[1] New York State Jour. Med., 1907, **7**, 177. (With good bibliography.)
[2] Jour. Inf. Dis., 1907, **4**, 171.
[3] Am. Jour. Path., 1927, **3**, 225.
[4] Parasit., 1913, **5**, 262.

Historical Note.—Notwithstanding the fact that syphilis is one of the oldest diseases known and studied, its cause was not discovered until the Treponema pallidum was discovered by Schaudinn and Hoffmann[1] in 1905.

Before this it was thought that the bacillus described by Lustgarten (1884) and others as occurring in small numbers in the lesions of syphilis bore an etiological relationship to the disease, but there were no evidences to support this view. Many other bacteria and several protozoan-like bodies have been erroneously regarded as the probable cause of syphilis.

Schaudinn announced early in 1905 that working with Hoffmann he found in the fresh exudates of chancre a spiral organism possessing characteristics similar to those of the spirochetes and he named it Spirocheta pallida. Later he concluded that this organism was individual enough (that is, it showed no undulating membrane, but possessed flagella) to be placed in a separate genus, which he named spironema. This name was found to be already in use so it was changed to Treponema. He thought that the organism was the cause of the disease. Extensive studies on human syphilis and on experimental syphilis in lower animals abundantly corroborated the work of Schaudinn and Hoffmann.

FIG. 163.—The two spirochetes in the center are Tr. pallidum; the three others, Sp. refringens. (Schaudinn and Hoffmann.)

FIG. 164.—Treponema pallidum appearing as bright refractive body on a dark field as shown by India ink or ultramicroscope.

The Organism (Fig. 163).—The Treponema pallidum is a very delicate structure closely resembling in morphology and staining reactions the *Treponema dentium*. It is somewhat longer, 4μ to 20μ long (average 10μ), and thinner, $\frac{1}{4}\mu$ to $\frac{1}{2}\mu$ in diameter. It has three to twenty sharp, deep spirals. The relationship between the length and the depth of the spirals is different in the two species; in *Treponema pallidum* length is to depth as 1 is to 1–1.5 (1μ long and 1μ to 1.5μ deep), while in *Treponema dentium* the average relationship is 1 to 0.5, the spirals being more shallow. The angle of the spiral turn is very sharp in both forms (more than 90°).

[1] Arbeit. a. d. Kaiserl. Gesundh., 1905, vol. **22.**

Flagella-like anterior and posterior prolongations are often seen in the *pallidum* but no definite flagella can be demonstrated. Schaudinn states that the division occurs very quickly (hence the reason why so few division forms are seen in stained preparations) and that it may be followed only by the most experienced observers during life. In the living condition the organism is not very refractive, so it is seen at first with difficulty. Its characteristic movements are rotation on its long axis, quivering movements up and down the spiral which is comparatively rigid, slight forward and backward motion and bending of the entire body. By the use of the ultramicroscope the motility of the organism is clearly seen (Fig. 164). Such a dark-field examination is especially useful in the early diagnosis of an untreated uninfected lesion. A negative result is presumptive proof that the lesion is not syphilis. (See p. 73 for procedure in dark-field examination).

Stages in a cycle of development have been described by Levaditi and others.[1]

Examination in Fixed Preparations.—The staining methods that have given the most satisfactory results may be found in Chapter III. The spirochetes on the whole take all stains faintly, but they may be clearly demonstrated in smears by the India-ink method (Fig. 164) and in sections by the silver-impregnation method, page 88.

Cultivation.—In 1907 Levaditi and McIntosh[2] obtained impure cultures of spirochetes in collodion sacs containing human serum and syphilitic material and placed in the peritoneal cavity of a monkey (*Macacus cynomolgus*). Schereschewsky[3] reported that he had obtained impure cultures of a spirochete from syphilitic lesions and blood in the following culture medium: horse serum sterilized by heat (58° to 60° C.) until it is of jelly-like consistency, and afterward autolyzed at 37° C. for three days. A piece of tissue excised from the lesion (*e. g.*, base of a papule or part of a lymph node) is inoculated into this medium, and grown at 37° C. The culture begins in three days, but the optimum is reached in five to twelve days.

Mühlens (1909) and later Hoffmann (1911) reported that they had also obtained cultures of a pallidum-like spirochete from syphilitic lymph nodes, grown at first in Schereschewsky's medium and afterward transplanted to broth and grown anaërobically. Animal experiments were negative.

Levaditi and Stanesco about the same time reported growing two species of spirochetes from a case of balanitis. One is a new one, which they found very like pallidum, but non-pathogenic for monkeys, and which they named *S. gracilis;* the other *S. balanitidis*. They employed as media (1) collodion sacs in tubes of fluid horse serum; (2) horse or human serum heated to 75° C. These spirochetes were never obtained in pure culture.

Noguchi (1911) obtained pure cultures from syphilitic lesions in the

[1] Am. Inst. Past., 1928, **42**, 475.
[2] Ann. de l'Inst. Past., 1907, **21**, 784.
[3] Centralbl. f. Bakt., 1908, **45**, 91.

rabbit, and later from human beings. His culture medium, contained in deep tubes, was a mixture of 1 part of ascitic or hydrocele fluid and 2 parts of 2 per cent. agar, in which was placed a small portion of sterile rabbit's kidney or other organ. The medium was covered by a deep layer of albolene. The spirochetes inoculated along a central stick grow out into the medium as a diffuse layer after ten days at 33.5° C., while most contaminating bacteria remain in the line of inoculation. Subcultures made away from the contaminating growths finally become pure. Noguchi obtained syphilitic papules by scarifying monkeys (*cercopithecus* and *macacus*), and inoculating his cultures. Such monkeys' blood gave a positive Wassermann reaction.

Zinsser and Hopkins[1] as well as others corroborated this work of Noguchi. Gilbert found that later pure cultures of spirochetes grow abundantly when slant egg media are used instead of kidney tissue. They also grow well in chopped meat medium.

Resistance of the Organism Outside the Body.—Mixed cultures can live in different light and at room temperature for eleven and one-half hours, while drying kills them within one hour (Zinsser and Hopkins.)

Pathogenesis.—So far as is known, syphilis spontaneously appears only in man. Since 1879, when Klebs stated that he had produced syphilis in monkeys by the inoculation of human virus, various experimenters have reported its transmissibility to these animals by direct inoculation. Most of the earlier reports did not state the exact identity of the animals employed nor did they give details of methods and results.

Metchnikoff and Roux, in 1903, produced a typical chancre on the genital mucosa of the young chimpanzee twenty-six days after inoculation. The essential lesion was followed by inguinal adenitis, and thirty days later by a generalized papular eruption. The virus was transferred in this case to lower monkeys. Most monkeys developed a primary lesion only, but some had abundant secondaries.

Since the discovery of the Tr. pallidum, experiments on monkeys have been more numerous and have been followed by more helpful results. More has been learned about the course of the infection in man, the evidence in favor of the Tr. pallidum being the cause of the disease has been strengthened, and many interesting investigations in regard to immunity have been made. Usually inoculations made by scarification on eyebrows or genitals are successful. The primary lesions are seen generally about thirty days after infection. In somewhat more than half the cases, after a slightly longer period, secondary symptoms (squamous papules on skin, and mucous patches in mouth) show themselves. No tertiary symptoms have been observed. Rabbits were first successfully inoculated by Hansell, then by Bertarelli.[2] The eye and the testes are favorable localities. Generalized syphilis can be produced in young rabbits by intracardiac inoculation (Uhlenhuth and Mulzer[3]).

Important features in regard to course of the infection were summarized by Ewing.[4]

The virus is not filtrable, though the spirochetes can grow through the pores of Berkefeld filter V and N in about four days (Noguchi). It is readily destroyed by heat (52° C.).

[1] Jour. Exp. Med., 1915, **21**, 576; 1916, **23**, 323, 341; **24**, 561.
[2] Centralbl. f. Bakt., 1906–1907, **41**, 520, 639; **43**, 167, 328.
[3] Berl. klin. Wchnschr., 1910, **47**, 1169.
[4] New York State Jour. Med., 1907, **7**, 177.

Luetin.—Noguchi found that an extract from his cultures, which he named luetin, gives a characteristic cutaneous reaction in syphilitic infection. In normal persons there is a slight local erythema, with possibly a small papule on the second day. In syphilitics there are three types of positive reaction:

1. Papular type. Large indurated red papule which increases for about four days.
2. Pustular type. The papule changes first into a vesicle and then into a pustule.
3. Torpid type. After a ten-day latent period a small papule forms which changes into a pustule.

These observations of Noguchi have been corroborated by Cohan and Robinson.

Symptoms in Man.—The course of the disease is divided into three stages: primary, secondary, and tertiary. The general character of the lesions in these stages is a more or less circumscribed formation of new tissue which is largely made up of small spheroidal cells alone or accompanied by a few endothelioid cells, and occasional giant cells.

The initial or primary lesion occurs in the form of a papule which develops into the so-called chancre, an ulcer with hardened base. Following this there is hyperplasia of the nearest lymph nodes. These lesions subside and six or seven weeks later the secondary lesions appear in various general eruptions on skin and mucous membranes and in other constitutional disturbances. The tertiary lesions which consist principally of the masses of new tissue called gummata are found throughout the viscera and in the periosteum.

Schaudinn's spirochetes have been demonstrated in all the lesions of syphilis (they are most easily demonstrated in the primary and secondary lesions), including the congenital types.

Immunity.—Natural immunity in syphilis is very peculiar. After the development of the primary lesions, man is usually insusceptible to reinoculation during the active stage of the disease, but during all stages both man and monkey can, in some cases, be reinoculated. Reinoculation in the tertiary stage gives precocious lesions of the tertiary type, gummata, and tubercles. Neisser found reinoculation from twenty-four to one hundred and four days after primary inoculation in monkeys sometimes effective, more often negative. During the stage when the skin is refractory to inoculation secondaries develop, showing that there is no complete immunity of the skin to the virus, since the *Treponema* is abundantly present in the lesions. Neisser suggests that cutaneous secondaries develop at periods of relative deficiency of immunity. He has shown that failure of reinoculation is not due to immunity to foreign infection and susceptibility to autoinfection, since the patient's own virus in both man and monkey is ineffective.

Many attempts to produce active immunity artificially have failed. Zinsser, Hopkins and McBurney[1] state that they can obtain from rabbits, which have been inoculated with repeated doses of these culture spirochetes, sera with antibodies (agglutinin) for their non-virulent cultures of *Tr. pallidum* but not for the recently isolated virulent strains.

[1] Jour. Exp. Med., 1915, **21**, 576; 1916, **23**, 323 341; **24**, 561.

Passive Immunization.—Injection of large quantities of serum of syphilitics into chimpanzees has failed to produce definite immunity, neither has the serum of animals repeatedly inoculated with syphilitic virus or with pure cultures of the pallidum had any prophylactic effect against the virus.

The Wassermann Reaction.—Wassermann, Neisser, and Bruck were the first to apply the Bordet-Gengou phenomenon to the diagnosis of syphilis. According to most workers, enough work has been done since then to establish its value as a diagnostic test. Some interesting points have been brought out in connection with this study, and many different methods have been recommended. (See Section on Wassermann Reaction for full discussion of subject, also for the Kahn precipitin test.)

Colloidal Gold Test.—In 1912 Lange[1] applied to spinal fluids the principle brought out by Zsigmondy (1901) that solutions of either electrolytes or of proteins precipitate colloidal gold, but that when both electrolytes and proteins are present the gold is not precipitated. Miller and his co-workers[2] were able to standardize the reagent, so the test became more reliable.

The test is simply another aid in helping to detect certain central nervous system involvements not responding so specifically to other tests. The test consists of a series of color changes in graded dilutions of spinal fluid, varying from negative salmon-red, then red-blue, blue, blue-gray, to colorless. Miller has worked out a "paretic curve."

Spirochetes in Frambesia Tropica (Yaws).—Castellani,[3] in 1906, announced that he had found in yaws a spiral organism which is called *Treponema pertenue*. He determined that monkeys (*macacus, semnopithecus*) are susceptible to inoculations with material from yaws patients apparently containing only this spirochete. Such material filtered is inert. He states that monkeys successfully inoculated with yaws do not become immune for syphilis, neither do those having had syphilis become immune for yaws. Castellani further states that specific characteristics between the two diseases are also brought out by means of the Wassermann reaction. His work has been corroborated by several observers. Frambesia lesions similar to those produced by syphilis in the testicles of rabbits were obtained by Nichols.[4] Levaditi and Nallan-Larrier[5] state that monkeys infected with syphilis are refractory to yaws, while those infected with yaws are susceptible to syphilis; therefore they conclude that yaws is a mild form of syphilis. The treatment of this condition by Castellani's mixture has given good results.[6]

Spirochetes in Infectious Jaundice.—Inada, Ido[7] and their collaborators in Japan, and Heubner and Reiter in Germany reported the presence of spirochetes (*Leptospira ictohemorrhagicæ*) in infectious jaundice

[1] Berl. klin. Wchnschr., 1912, **49**, 897; Ztschr. f. Chem., 1913, **1**, 44.
[2] Bull. Johns Hopkins Hosp., 1914, **25**, 123; 1915, **26**, 391.
[3] Jour. Hygiene, 1907, **7**, 558.
[4] Jour. Exp. Med., 1910, **12**, 616; 1911, **14**, 196.
[5] Ann. de l'Inst. Past., 1908, **22**, 260. [6] Phila. Jour. Sc., 1918, Sec. D, p. 89.
[7] Jour. Exp. Med., 1916, **23**, 377, 557; **24**, 471.

or Weil's disease. This work was confirmed by Stokes and by Noguchi. Ido and his co-workers found the same species in both house and field rats, and obtained active immunity in guinea-pigs against the organism. Noguchi[1] considered this a new genus and suggested the name *Leptospira* for it. He reported finding the same species in the rats captured in New York City and vicinity. Jobling and Eggstein[2] reported finding a similar spirochete in the wild rats of the Southern States, and Otteraaen[3] found a similar spirochete in the rats about Chicago. In 1921 a number of cases of infectious jaundice occurred in New York State. A study made of these cases including a case of accidental infection of one of the workers by Leptospira ictohemorrhagicæ is reported by Wadsworth and co-workers.[4] Inada and others recommend intravenous serotherapy.

Ido, Ito and Wani found a similar spirochete in "seven-day fever" which Noguchi has called Leptospira hebdomalis. It is carried by the field mouse.

Spirochetes in Yellow Fever.—Stimson in 1917 first described finding a spirochete in the blood of yellow fever patients. He called it interrogans because of its appearing so often in the shape of an interrogation point. When Noguchi in 1919 also found spirochetes in yellow fever he decided that they belonged to his new genus and called them Leptospira icteroides. Later, after studying Stimson's spirochete, he came to the conclusion that it was the same as his, so the name became Leptospira interrogans. The evidence Noguchi so painstakingly gathered to establish an etiological relationship between this spirochete and yellow fever has not been corroborated.

Spirochetes in Rat-bite Fever.—Futaki and his associates[5] have reported the presence of a spirochete in cases of rat-bite fever in Japan. They place it among the treponemata.

BORRELIA RECURRENTIS AND ALLIES.

Borrelia recurrentis (**Spirillum obermeieri**) was first observed by Obermeier in 1873 in the blood of persons suffering from relapsing fever. It was found in large numbers during the height of the fever, it disappeared about the time of the crisis, and reappeared during the relapses. It was not found in other diseases. Obermeier considered it the cause of the disease, and his views were shown to be correct by the production of the disease in man and ape through experimental inoculation.

Morphology.—The organisms are long, slender, flexible, spiral or wavy filaments, with pointed ends, from 16μ to 40μ in length and from one-quarter to one-third the thickness of the cholera spirillum ($2\frac{1}{2}\mu$ to $\frac{3}{4}\mu$). Novy[6] and others have described a terminal flagellum (Fig. 165).

[1] Jour. Exp. Med., 1917, **25**, 755, and 1918, **27**, 575.
[2] Jour. Am. Med. Assn., 1917, **69**, 1787.
[3] Jour. Inf. Dis., 1919, **24**, 485.
[4] Jour. Am. Med. Assn., 1922, **78**, 1120.
[5] Jour. Exp. Med., 1916, **23**, 249.
[6] Jour. Inf. Dis., 1906, **3**, 291.

Some investigators, however, believe the organism has merely tapering ends and no flagellum. They possess three to twelve wide, more or less irregular spirals. They stain somewhat faintly with watery solutions of the basic anilin dyes, better with Löffler's or Kühne's methylene-blue solutions, or with carbol fuchsin; best with the Romanowsky method or its modifications. They are negative to Gram.

Biological Characteristics.—In fresh preparations from the blood the spirochetes exhibit active progressive movements, accompanied by very rapid rotation in the long axis of the spiral filaments or by undulating movements. They are found only in the blood or blood organs, never in the secretions; and they appear during the fever, not in the intermissions, or at most singly at the beginning of an attack, or for a short time afterwards.

FIG. 165.—Photograph of Bor. recurrentis showing terminal flagellum. × 3000 diameters. (After Novy.)

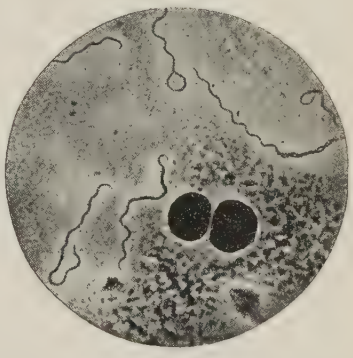


FIG. 166.—Bor. recurrentis blood-smear. Fuchsin. × 1000 diameters. (From Itzerott and Niemann.)

When kept in blood serum, or a 0.6 per cent. solution of sodium chloride, they continue to exhibit active movements for a considerable time. They may be preserved alive and active for many days in sealed tubes. They are killed quickly at 60° C., but they remain alive for some time at 0° C. Unsuccessful efforts to cultivate them in artificial culture media have been made from time to time. Koch has observed an increase in the length of the spirilla and the formation of a tangled mass of filaments. Novy finally succeeded in cultivating them in celloidin capsules placed in the peritoneum of rats. His culture remained virulent (with a slight loss) for many generations. Noguchi[1] and others also cultured them by different methods.

Pathogenesis.—In man, whether the disease is acquired naturally or by artificial inoculation, the organism causes the following symptoms. After a short period of incubation the temperature rises rapidly, remains high for five to seven days, and then returns to normal by crisis. About

[1] Jour. Exp. Med., 1913, vol. **17.**

seven days later there is another sudden rise of temperature, but this time the crisis occurs sooner. A second or third relapse may occur. The organisms increase in numbers rapidly in the blood from the beginning of the fever, large numbers often being found in every microscopic field. They begin to disappear a short time before the crisis, and immediately after the crisis it is practically impossible to find them in the circulating blood. The mortality varies in different epidemics from 2 to 10 per cent. When monkeys are inoculated with human blood containing the spirilla, they become sick about three and a half days later, but show only the initial febrile attack or, at the most, an occasional short relapse. The organisms are found to have the same relation to the pyrexial periods as in man. Blood from one animal taken during the fever induces a similar febrile paroxysm when inoculated into another animal.

Metchnikoff showed that during the intermissions when the spirochetes disappeared from the circulating blood they accumulated in the spleen and were ingested in large numbers by certain phagocytes and finally were destroyed.

According to Lamb, a certain amount of immunity is conferred upon monkeys (*Macacus radiatus*) soon after an attack, but it disappears quickly. If the serum is removed during this time it is found to have some protective action when mixed with the blood containing spirilla and also to cause agglutination of the organisms. Novy (1906) showed that a powerful specific germicidal body exists in the blood of rats during and after recovery, notably in the blood of hyperimmunized rats. An immunizing body probably distinct from this is also present. He also showed that passive immunity can be imparted by injections of recovered or hyperimmunized blood, that both active and passive immunity may last for months, and that the serum has both a preventive and a curative action. Agglutinins are also present in such a serum.

Breinl has shown that the immunity produced by *S. obermeieri* does not protect against *S. duttoni*, and *vice versa*. Neither does it protect against the Asiatic or American variety of this type of spirochete.

The strain found in Bombay seems to be more virulent than that in Europe.

Borrelia Duttoni.—The organism shown by Dutton (1905) to be the cause of African tick fever is very similar morphologically to *S. obermeieri*, but Novy, Fränkel, and others have shown slight differences which make them believe that it is another variety, if not another species of this group. Leishman[1] gave a good review of the investigations to date on this spirochete.

Borrelia Carteri.—This spirochete was described by Carter in 1887 as causing relapsing fever in Bombay. Monkeys were inoculated by Carter successfully with the human blood containing this spirochete.

Borrelia kochii (1906) found in some cases of African fever, Borrelia novyi (1906) found in American relapsing fever, Borrelia berbera (1908)

[1] Parasitology, 1911, 4, 133 and 183.

in still other cases of fever in Africa, and a number of other strains have been reported all with similar morphology, but different serological properties.

Filtrability of Borrelia.—A number of workers have demonstrated that Borrelia may pass through Berkefeld's N and V under pressure.

INSECT CARRIERS OF SPIROCHETES AND ALLIES.

These microörganisms may be conveyed by the bites of blood-sucking insects or arthropods. Many studies have been made on this subject since the work of Marchoux and Salimbeni (1903) on fowl spirochetes, and of Dutton and Todd (1905) on African tick fever. Nuttall and his associates added much to our knowledge of ticks and other arthropods as vectors. Dutton and Todd demonstrated that *Bor. duttoni* can be transferred to monkeys by the bites of young ticks (*Ornithodirus moubata*) at their first feed after hatching from infected parents. Dutton accidentally demonstrated the fact that the disease can be inoculated into human beings through a cut surface, for after a wound received at autopsy he developed the disease which caused his death.

Leishman (1909) demonstrated that the second generation of ticks may also infect. He was unable to find spirochetes in tissues of ticks later than ten days after feeding, though young ticks from eggs hatched after this time were infective. He found, however, granules which he considered changed spirochetes. Hindle agreed with him. The fact that ticks may be infective long after the disappearance of typical spirochetes, if they are placed at 30° to 35° C., is evidence of the infectious nature of these granules. Möller found that a tick may remain infective for a year and a half or more after its initial feed from an infected host. Möller also showed that the third generation of ticks might inherit infection. Mackie obtained strong evidence that the body louse is carrier of *Bor. recurrentis*. His observations have been corroborated by a number of observers.

Nicolle and his colleagues found after twenty-four hours spirochetes disappear from gut of the louse (*P. vestimenti*) to reappear in from eight to twelve days and continue for eleven days and possibly longer. Monkeys inoculated with the contents of lice fifteen days after feeding develop relapsing fever.

CHAPTER XXXVI.

PATHOGENIC MICROÖRGANISMS BELONGING TO THE HIGHER BACTERIA (TRICHOMYCETES).

(See Part I For General Descriptions.)

Leptotrichia Infections.—Leptotrichia forms growing anaërobically are frequently found in the human mouth (*Leptotrichia buccalis*), and a few writers have claimed that under certain conditions these may become pathogenic, but since no corroborative work has been done, no opinion can be formed of the worth of these observations. The few reports of aërobic cultural forms[1] do not describe characteristics minutely enough to make them distinctive.

ACTINOMYCES.

(Including All Forms called Nocardia or Streptothrix, see Classification, p. 40). Also Large Table at end of Part I.

The first definite description of a disease produced in animals by a filamentous bacterium was given by Bollinger in 1877, though the little clumps produced by this group of parasites were first seen by von Langenbeck in 1845. Bollinger found the characteristic ray-like clumps in a farcy-like disease of the ox. At his request the organism was studied by the botanist Harz who gave it the name of actinomyces, or ray fungus. It was reported in human beings by Israel in 1878.

A number of other investigators described diseases in humans as due to this type of organism, but few of them obtained the organism in pure cultures, and the descriptions of those who did are incomplete. Among the earlier investigators who studied the cultural forms more minutely were Boström (1890), and Wolf and Israel (1891).

The characteristics of the microörganisms described by Boström[2] and those by Wolf and Israel differed greatly. Boström's organism grew best aërobically and developed well at room temperature. He noted the intimate relation of the organism to those on fragments of grain, and this led to the finding of similar microörganisms in the outer world on grains, grasses, etc. There is no doubt that some suppurative processes have been due to organisms having these characteristics, but they do not seem to excite true actinomycosis.

Wolf and Israel[3] described a microörganism from two human cases, which differs from that described by Boström, but agrees with the microörganisms obtained by most of the more recent investigators

[1] Gifford: Jour. Inf. Dis., 1920, **27**, 296.
[2] Beitr. path. Anat., etc., 1890, vol. **9**.
[3] Virchow's Arch., 1891, **126**, 4.

(notably Wright) from typical actinomycosis. It grew best under anaërobic conditions and did not grow at room temperature. Its growth was much less luxuriant than Boström's microörganism. On the surface of anaërobic agar slant cultures on the third, fourth and fifth day numerous minute, isolated, dew-drop-like colonies appeared, the largest pinhead in size. These gradually became larger and formed ball-like, irregularly rounded, elevated nodules varying in size up to that of a millet seed, exceptionally becoming the size of a lentil or larger. As a rule the colonies did not become confluent, and an apparently homogeneous layer of growth was seen to be made up of separate nodules if examined with a lens. In some instances the colonies presented a prominent center with a lobulated margin and appeared as rosettes. A characteristic of the colonies was that they sent into the agar root-like projections. In aërobic agar slant cultures no growth or a slow and a very feeble growth was obtained. In stab cultures the growth was sometimes limited to

Fig. 167.—Smear from bouillon culture of actinomyces. × 1500 diameters. (From Wright.)

the lower portion of the line of inoculation or was more vigorous there. In bouillon, after three to five days, growth appeared as small white flakes, partly floating and partly collected at the bottom of the tube. Growth occurred in bouillon under aërobic conditions, but was better under anaërobic conditions. The organisms grew in branching and interlacing filaments which later tended to break into segments (see Fig. 167). The microörganism in smear preparations from agar cultures appeared chiefly as short, homogeneous, usually straight, but also comma-like or bowed rods, whose length and breadth varied. In many cultures short, plump rods predominated, and in others longer, thicker, or thinner individuals were more numerous. The ends of the rods often showed oval or ball-like swellings. Swollen clubs were formed irregularly in the presence of blood or serous fluids.

On intraperitoneal inoculation guinea-pigs and rabbits after four to seventeen weeks showed, after being killed, tumor growths mostly

in the peritoneal cavity and in one instance in the spleen. Microscopic examination of the tumors showed in all cases but one the presence of typical actinomyces colonies, in most cases with typical "clubs." The general histological appearance of the tumors was like that of actinomycotic tissue.

Wolf in a later paper reports that an animal inoculated in the peritoneal cavity with a culture of the same organism had lived a year and a half. At the autopsy several tumors were found in the peritoneal cavity, and in the liver a large typical tumor in which were many colonies which by microscopic examination were shown to be typical club-bearing actinomyces colonies.

Wright,[1] in 1905, made an extensive study of actinomycosis and added greatly to our knowledge of it. The following description is taken mostly from his reports.

Naked-eye Appearance of Colonies of Parasite in Tissues.—In both man and animals they can be readily seen in the pus from the affected regions as small, white, yellowish or greenish granules of pinhead size (from 0.5 to 2 mm. in diameter). When pus has not formed they lie embedded in the granulation tissue.

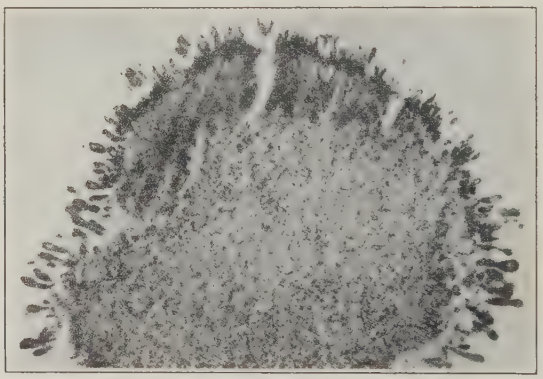


FIG. 168.—A typical "club"-bearing colony of actinomyces. × 325 diameters. (From Wright.)

Microscopic Appearance.—Microscopically these bodies are seen to be made up of threads which radiate from a center and present bulbous, club-like terminations (Fig. 168). These club-like terminations are characteristic of the actinomyces. They are generally arranged in pairs, closely crowded together, and are very glistening in appearance. They are more common in bovine than in human lesions. They have been thought to be reproductive elements, but they are probably simply a reaction of the filament end to the host tissue. The threads which compose the central mass of the granules are from 0.3μ to 0.5μ in diameter. The threads show true branching

[1] Jour. Med. Res., 1905, **7**, 349.

and in the older colonies show a segmentation which gives them the appearance of chains of cocci. Sometimes the whole center of the colonies seems to be a mass of coccus-like bodies most of which are considered spores or conidia; the clubs are from 6μ to 8μ in diameter.

The threads and spores are *stained* with the ordinary anilin colors, also by Gram's solution; when stained with gentian violet and by Gram's method the threads appear more distinct than when stained with methylene blue. The clubs usually lose their stain by Gram's method and take the contrast strain. Certain strains are relatively acid fast.[1]

Isolation of Actinomyces.—The aërobic strains are isolated with comparative ease. The anaërobic strains are grown with difficulty. A large number of solidified blood serum or serum agar tubes are inoculated with the hope that one or two will develop a growth. The cultures appear much like those of tubercle bacilli. They commonly grow, however, into the medium, and take on a yellowish hue. Wright recommends that granules, preferably obtained from closed lesions, be first thoroughly washed in sterile water or bouillon and then crushed between two sterile glass slides. (In bovine cases make sure the granule has filamentous masses, for, if not, no culture will grow.) The crushed granule is transferred to a tube of melted 1 per cent. glucose agar at 40° C. The material is thoroughly distributed by shaking and the tube placed in the incubator. A number of granules after washing should be placed on the inside of a sterile test-tube and allowed to dry. In this way, should the material be contaminated, the drying of the granules for several weeks may kill off the other organisms. The tube should be examined daily. If a number of living filaments were added to the agar a large number of colonies will develop. These will be most numerous in a zone 5 to 12 mm. below the surface (microaërophiles).

The cultures are quite resistant to outside influences; dried, they may be kept for a year or more; they are killed by an exposure of five minutes to a temperature of 70° C.

Experimental Inoculation in Animals.—True progressive infection is rarely or never obtained by the injection of pure cultures into rabbits, guinea-pigs, or other small animals. In cattle, however, the disease has been produced from cultures. The cultures form the characteristic "club"-bearing colonies in the tissues of experimental animals. These colonies are either enclosed in small nodules of connective tissue or are contained in suppurative foci within nodular tumors made up of connective tissues in varying stages of development.

Occurrence.—Actinomycosis is quite prevalent among cattle, in which it occurs endemically; it is more rare among swine and horses. A number of cases have been reported in man. Sanford and Magath[2] reported 96 cases seen in the Mayo Clinic, also 119 cases collected from literature. New and Figi[3] stated that 127 cases with actinomycosis were examined in the Mayo Clinic from 1910 to 1921, in only 3 of which it had affected the tongue, though this is a common site in cattle. The disease is rarely communicated from one animal to another and no case is known where a direct history of human contagion has been obtained. The cereal grains, which from their nature are capable

[1] Henrici and Gardner: Jour. Inf. Dis., 1921, **28**, 232. (Bibliography.)
[2] Minnesota Med., 1922, **5**, 71.
[3] Am. Jour. Med. Sci., 1922, **163**, 507.

of penetrating the tissues, have been found in centers of actinomycotic infection in the lower animals. The microörganism may also be introduced by means of carious teeth. Cutaneous infection has been produced by wood splinters, and infection of the lungs by aspiration of fragments of teeth containing the fungus. The presence of the microorganism in cereal grains is denied by Wright and therefore placed in doubt. The further distribution of the fungus after it is introduced into the tissues is effected partly by its growth and partly by conveyance by means of the lymphatics and leukocytes. Not infrequently a mixed infection with the pyogenic cocci occurs in actinomycosis.

Characteristics of Disease in Man and Animals.—In the earliest stages of its growth the parasite gives rise to a small granulation tumor, not unlike that produced by the tubercle bacillus, which contains, in addition to small round cells, epithelial elements and giant cells. After it reaches a certain size there is great proliferation of the surrounding connective tissue, and the growth may, particularly in the jaw, look like, and was long mistaken for, osteosarcoma. Finally, suppuration occurs, which, according to Israel, may be produced directly by the fungus itself.

The course of the disease is usually very chronic. Commonly the first sign is a point of infiltration about the lower jaw or lower on the neck. This almost painless swelling increases and finally softens in its center. The necrotic tissue finally forces a passage externally or, passing downward, infects the pleura, lungs, mediastinum or ribs. As a rule, the disease is not accompanied by fever. In cattle the disease is usually situated in some portion of the head, especially in the jaw, tongue or tonsils, hence called lumpy jaw, wooden tongue, etc. Primary lung, intestinal and skin lesions are not infrequent. These local lesions sometimes scatter and produce a general infection and the udder may be involved.

Treatment.—In 1892 Nocard showed that the disease in animals might be cured by iodide of potassium, calling attention to the fact that Thomassen had recommended this treatment in 1885. It is given in doses of $1\frac{1}{2}$ to $2\frac{1}{2}$ drams once a day. Salmon and Smith (U. S. Bureau of Animal Industry, Circular No. 96) give directions as to its use.

Strains from Cases Called Nocardiosis by Some (Wright) and Streptothricosis by Others.—From widely scattered localities and at long intervals of time reports have been published describing unique cases of disease produced by varieties of microörganisms belonging to the branching bacteria. In some of these cases, points of similarity can be recognized in the clinical symptoms and the gross pathological lesions, while others differ widely in both respects. Thus this type of organism has been described as occurring in brain abscess, cerebrospinal meningitis, pneumonic areas, and in a few other pathological conditions. Eppinger[1] who was one of the first to describe an organism of this character, injected cultures into guinea-pigs and rabbits, and observed that they caused lesions similar to tuberculosis. He called his organism a cladothrix, but it is now classed as an actinomyces. Consolidation of portions of both lungs, thickening of the peritoneum, and scattered nodules resembling tubercles were noted by Flexner[2] in a case of human

[1] Wien. klin. Wchnschr., 1890.
[2] Jour. Exp. Med., 1896, vol. **3**.

infection due to a thread-forming organism in which the pathological picture of the disease resembled so nearly that of tuberculosis in human beings that the two diseases could be separated only by finding the causative microörganism in each case. But in no two cases reported up to the present time have the descriptions of the microörganisms found agreed in all particulars. In some cases no attempt at cultivation was made. In other cases numerous and careful plants on various culture media failed to develop the specific organism. As late as the year 1904 Tuttle[1] was able to find the reports of only 12 cases in which "nocardia" was found in sufficient abundance to have been an important, if not the principal, factor in producing disease. These cases were all fatal, and only once was the character of the disease recognized during life. As the clinical symptoms and the lesions in the human subjects as well as in the animals experimentally inoculated with nocardia often resemble those of miliary tuberculosis, the question is naturally suggested whether cases of nocardia tuberculosis are not more numerous than the few reported cases would indicate. The most universal prevalence of genuine tuberculosis and the extreme gravity of the disease have so long occupied the attention and study of the medical profession that much is taken for granted, and in cases in which the symptoms and lesions resemble with some closeness those characteristic of the well-known disease they may easily be set down without question to the account of the tubercle bacillus. The cases of "nocardiosis" ("streptothricosis," actinomycosis) reported which simulated tuberculosis have been fatal, and the lesions for the most part have been widely distributed, but in a number of cases old lesions have been found which suggest that the disease may have been localized for a longer or shorter time, and then, by some accident, may have become rapidly general. In this respect also these cases may resemble tuberculosis.

As the lungs have appeared to be the seat of the primary infection in many of the cases of human actinomycosis it is very desirable that all cases presenting the physical signs of tuberculosis, in which repeated examinations fail to discover the tubercle bacillus, should be systematically examined for threads. In this way the frequency of the disease may be determined. Gram's method of staining is one of the most reliable agents for demonstrating these organisms.

Typical Clinical Course of Acute Lung Infection (Tuttle's case[1]).—Illness began with chill and fever and pain in side and back. The pain in the side became worse and breathing became difficult. Cough and slight expectoration followed. At irregular intervals alternating hot and chilly sensations occurred.

The physical signs indicated an attack of acute lobar pneumonia, the area of consolidation being small and situated in the lower part of the left upper lobe in front. Frequent and violent coughing, with almost no expectoration, pain in the affected side and in the lumbar region, restlessness and sleeplessness and involuntary urination are the symptoms noted during the first four days in the hospital. The pneumonic area increased somewhat and extended backward to the posterior axillary line, and the temperature was continuous at 103° to 103.5°. On the fifth day the temperature fell 2° and signs of reso-

[1] Med. and Surg. Rep. Presbyterian Hospital, New York, 1904, 6, 147.

lution appeared in the consolidated area. The apparent improvement, however, was of short duration. On the sixth day the temperature rose to 104.5°, and continued to rise each day, reaching 107.5° shortly before death, which occurred on the ninth day in the hospital and the fifteenth day of the disease. There were repeated attacks of profuse sweating. On the day before death three indurated swellings beneath the skin were noticed. One, on the left forearm, about the size of a walnut, apparently contained pus. Two, of smaller size, were situated in the right groin.

Blood cultures from a vein in the arm, taken on the sixth day, remained sterile. The leukocyte count on the seventh day was 36,000.

Pathological Findings.—On the right arm, the left forearm, the abdominal wall and on both thighs there were eight to ten slightly projecting, rounded, fluctuating, subcutaneous swellings from $\frac{1}{2}$ to 1 inch in diameter. The nodules were composed of bluish-gray, thick, mucilaginous matter, which was very tenacious and could be drawn out into long threads. The lower lobe was thickly studded with miliary tubercles, and scattered through the entire lung were suppurating foci. Liver and spleen normal. Kidneys: The surface was evenly dotted with minute white spots, which suggested septic emboli rather than tubercles. A few prominent white nodules, from $\frac{1}{4}$ to $\frac{1}{2}$ inch in diameter, contained thick, tenacious matter (Fig. 169). Section showed that the entire substance of the kidney was densely studded with these minute white granules.

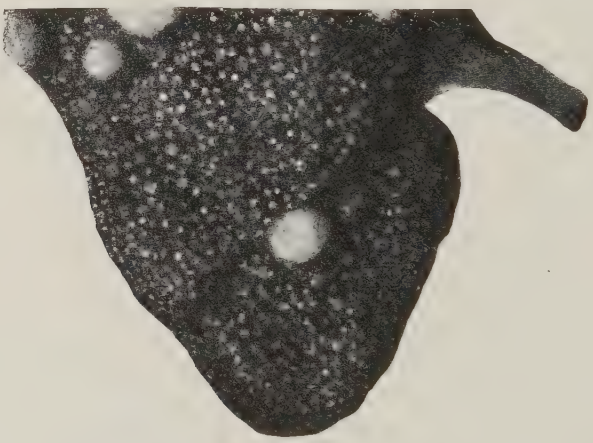


FIG. 169.—Portion of kidney showing minute and large areas of infection.

The gross pathological conditions were interpreted before "nocardia" was found as follows: An old tuberculous nodule in the right lung; acute miliary tuberculosis in the right lung and peritoneum; acute lobar pneumonia, affecting the left lung; septic infarctions and pyemic abscesses of both lungs, heart muscle, both kidneys, pancreas, mesenteric lymph nodes, and subcutaneous connective tissue. The miliary tubercles of the right lung and peritoneum presented the characteristic appearance of genuine tuberculosis. They were minute, hard, gray, almost translucent nodules, while the granules in the kidneys were of an opaque white or yellowish-white color.

Microscopic Examination.—Smears from the abscess beneath the skin and on the surface of the kidneys, stained with methyl blue, carbol-fuchsin and by Gram's method, showed a large amount of mucoid material containing a number of leukocytes and an occasional irregularly curved, thread-shaped microorganism. The more slender threads were evenly stained, but some fragmentation or beading of the protoplasm could generally be observed. The thicker threads and broken fragments showed deeply stained globules and irregular

bodies in a faintly visible rod or thread-shaped covering. Some branching threads were observed. No other microörganisms were found in the smears. Stained by Gram's method, with care not to decolorize too completely, threads like those described in the abscesses were found in great abundance, but rather faintly stained throughout the tuberculous-like areas in lungs. No threads could be found within the typical tubercles with giant cells, but in the zones of small cells around them they were seen in great numbers, winding about among the cells and forming a sort of network. No other microörganisms could be found except in the pneumonic area of the left lung, where some groups of cocci were seen.

Culture Experiments.—Tubes of Löffler's blood serum were inoculated from the kidneys and kept at 37° C. On the third day minute white colonies appeared in some of the tubes, and on the fifth day all the tubes (6) showed from three to ten or twelve similar colonies in each. The colonies increased in size until some of them reached a diameter of $\frac{1}{8}$ inch. The color, at first white, changed to yellowish-white and then to a decided pale yellow. The well-developed colonies clung firmly to the surface of the medium and were not easily detached or broken up. The growths in all of the tubes were absolutely pure, and consisted of branching threads like those found in the sections.

Löffler's blood serum or other blood medium seems to be the most suitable for cultures.

On plain agar and glycerin agar the growth was the same as on blood serum, but was less rapidly developed.

In bouillon the growth was slow. If the tube was not disturbed or jarred, minute white tufts were seen clinging to the surface of the glass. But if the tube was shaken even slightly they sank slowly to the bottom, forming a white fluffy layer. These growths when undisturbed resembled minute balls of thistledown. The yellow color was not apparent even in the mass at the bottom of the tube. It was strictly aërobic.

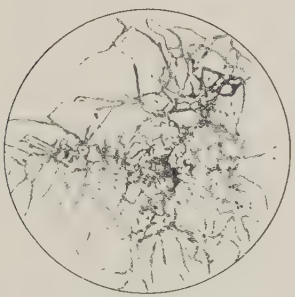


Fig. 170.—Streptothrix from bouillon culture. (From Tuttle.)

Fig. 171.—Young streptothrix threads showing terminal buds. (From Tuttle.)

Morphology (Figs. 170 and 171.)—On blood serum the threads were comparatively thick and coarse, but those growing in bouillon were very slender and delicate. The main trunk also was often thicker than the branches. When unstained they were homogeneous gray threads, without any appearance of a central canal or double-contoured wall. There was never any segmentation of the threads. When properly stained there was always a distinct beading or fragmentation of the protoplasm, but overstaining with fuchsin produced rather coarse, evenly stained rods. The branching was irregular and without symmetry, and the branches were placed at a wide angle, very nearly, and sometimes quite, at right angles. This was best seen in specimens taken from liquid media. The irregularly stellate arrangement of the branches, which was observed by Eppinger in his original specimen, was often seen in young organisms floated out from a liquid medium.

Spore Formation.—On examining the deep orange or red-colored growth upon potato, one was surprised to find that the threads had entirely disappeared and that the specimen consisted of moderately large coccoid forms. These represent the spore form of the organism, and when planted upon blood serum the branching threads again appear. The spores stained readily with carbol-fuchsin and were not easily decolorized. They were spherical, or nearly so, but often appeared somewhat elongated, apparently from beginning germination. They were killed by exposure to moist heat of 65° to 70° C. for an hour, but were more resistant to dry heat. Drying destroyed the threads after a comparatively short time, but the spores retained their vitality for an indefinite period. A dried-up potato culture retained its vitality at the end of almost four years.

Animal Inoculations.—A number of rabbits and guinea-pigs were inoculated subcutaneously upon the abdomen and in the neighborhood of the cervical, axillary and inguinal lymph nodes with colonies broken up in salt solution. Indurated swellings were produced at the point of inoculation and a number of abscesses resulted. The abscesses developed rapidly and some of them opened spontaneously, while others were incised. The material evacuated did not resemble ordinary pus, but was thick and mucilaginous and exceedingly tenacious, like that from the subcutaneous abscesses of the patient described above. The microscopic appearance was the same, and the threads were found in considerable numbers. Several rabbits and guinea-pigs and two cats received peritoneal inoculations, but none of them showed any sign of infection. When rabbits were inoculated intravenously, a rapidly fatal general infection was produced, and the lesions were similar in kind and distribution to those described in the human subject.

Other Cases Reported.—Ferré and Faguet found in Bordeaux, in a cerebral abscess in the centrum ovale, a branching fungus, colored by Gram, which corresponded to nocardia. It grew on agar in round, ochre-colored colonies; on potato there was little growth visible; slimy, tough colonies, which became gray and remained free from white dusting on the surface. Inoculations in rabbits and guinea-pigs were negative.

Neal working on Elmendorf's case of meningitis found a branching anaërobe.[1]

Varieties of branching bacteria have been found in the human vagina. We have found such a variety in several cases of stillbirth with invasion of the placenta with the same organism.

Numerous cases have been observed in which similar organisms proved to be the cause of chronic lung diseases, clinically suspected to be tuberculosis.

Snoke[2] reports several cases with different manifestations.

Blake,[3] Tunnicliff[4] and others have found streptothrices in the blood of patients with rat-bite fever. Dick and Tunnicliff[5] found them in the blood of a patient bitten by a weazel.

Pettit[6] reported an epidemic in oysters due apparently to an actinomyces-like organism.

[1] Elmendorf and Neal: Arch. Ped., April, 1925.
[2] Am. Jour. Med. Sci., 1928, **175**, 69.
[3] Blake: Jour. Exp. Med., 1916, **23**, 39.
[4] Tunnicliff and Mayer: Jour. Inf. Dis., 1918, **23**, 555.
[5] Ibid., **23**, 183.
[6] Bull. de l'Acad. de méd., 1921, **85**, 235.

Treatment.—Autogenous vaccines have been tried in certain lung cases, with varying results.[1] Potassium iodide in large doses should be given. *X*-rays and radium have also been reported as helping.

Mycetoma (Madura Foot).—This is a purulent inflammation of the foot occurring primarily in warm climates. The inflammation is accompanied by much irregular enlargement of the foot. Three varieties of this condition have been described based upon the color of the granules found in the diseased area: (1) white, (2) black, and (3) red. The white variety has been studied by Musgrave and Clegg[2] (1907) who have isolated an organism resembling somewhat actinomyces and somewhat the organism isolated by Wright (1898) from a black variety of the disease. Wright's is probably a true mould. Winslow[3] says that only 7 cases have been reported in the United States and Canada. Da Silva[4] described two other species of fungus from a case of mycetoma in Brazil.

Actinobacillus.—In a disease of cattle similar to actinomycosis Lignières and Spitz[5] found a ray-like growth in lesions, but obtained on cultures only a Gram-negative streptobacillus. Ravaut and Pinoy[6] reported similar findings in a human being.

Erysipelothrix.—In a disease in swine called swine erysipelas, a rod-shaped organism has been found. It has a tendency to form long filaments which may show branches. It is non-motile, Gram-positive, microaërophilic.

Klauder[7] has given a review of swine erysipelas in the United States and of erysipeloid, which is supposed to be the same disease, as it occurs in man.

[1] Colebrook: Lancet, 1921, **1**, 893.
[2] Phila. Jour. Sci., 1907, **3**, 2, 477.
[3] Amer. Surg., 1917, **66**, 496.
[4] Brazil Medico., 1919, **33**, 81.
[5] Bull. d. l. Soc. Cent. d. Med. Vet., Sept. 30, 1902.
[6] Presse méd., 1911, **19**, 49.
[7] Jour. Am. Med. Assn., 1926, **86**, 536.

CHAPTER XXXVII.

THE PATHOGENIC MOULDS (HYPHOMYCETES, EUMYCETES) AND YEASTS (BLASTOMYCETES).

THE HYPHOMYCETES.

The majority of the moulds are not pathogenic for human beings, and interest us more as organisms which are apt to infect foodstuffs and media. Some are, however, true parasites, and produce a number of rather common diseases; for example, ringworm, favus, thrush, and pityriasis versicolor. Certain of the commoner moulds (mucor, aspergillus) have also been reported from time to time as present in pathogenic conditions in man as well as in the lower animals. Many varieties have been found in plant diseases, and some may be a source of danger to man indirectly. Indeed, when they form poisonous substances, as in the infection of grain by *Claviceps purpurea* (ergot poisoning), they are distinctly dangerous.

Paltauf[1] reported the case of a man who died after enteritis with secondary peritonitis. The autopsy showed multiple abscesses in brain and lungs, besides the lesions in the intestines and peritoneum, in all of which a species of mucor was found. Two other cases of primary mucor infection in humans were reported by Furbringer. A number of species of mucor have been found in ear and eye infections; for example, Mucor corymbifer (Fig. 172) has been found in ophthalmia. A number of species of moulds are pathogenic for lower animals. Aspergillus[2] is found thus very frequently in lower animals, especially in birds, where a kind of pseudotuberculosis is often produced. Quite a number of similar cases have been reported in man, and it is supposed that the infection may be carried rom birds to man. Aspergillus nodulans (branched sterigmata) and Aspergillus fumigatus (Fig. 173) are the most frequent varieties found. Penicillium minimum (similar to glaucum, Fig. 174) has been found by Liebermann in inflammation of the ear. Mendelson[3] calls attention to the frequency of tropical broncho-pulmonary mycoses.

The more common pathogenic forms for man are those producing the various hair and skin lesions mentioned above. These moulds are all classed with the fungi imperfecti. The moulds described as causing "trench foot" were shown to be only secondary invaders.[4] (See p. 42 for general characteristics of moulds; see also Plate III.)

The Trichophytons (Tinea, Ringworm Fungus).—Ringworm of the body or hairless parts of the skin (called also tinea circinata) and ringworm of the hairy parts (called also tinea tonsurans, herpes tonsurans or sycosis) are due to several species of the imperfecti group.

[1] Virchow's Arch. Path. Anat., 1885, **102**, 543.
[2] Thom and Church: The Aspirgilli, Baltimore, Williams and Wilkins Co., 1926.
[3] Jour. Am. Med. Assn., 1921, **77**, 110.
[4] Sweet Norris and Wilmer: Lancet, 1918, **1**, 564. Wilson, B.: Internat. Am. Med. Museum Bull., 1918, No. 7, 202. Cobbett: War. Med., 1918, **2**, 707.

According to Sabouraud,[1] whose conclusions are based on an extensive series of microscopic examinations of cases of ringworm in man and

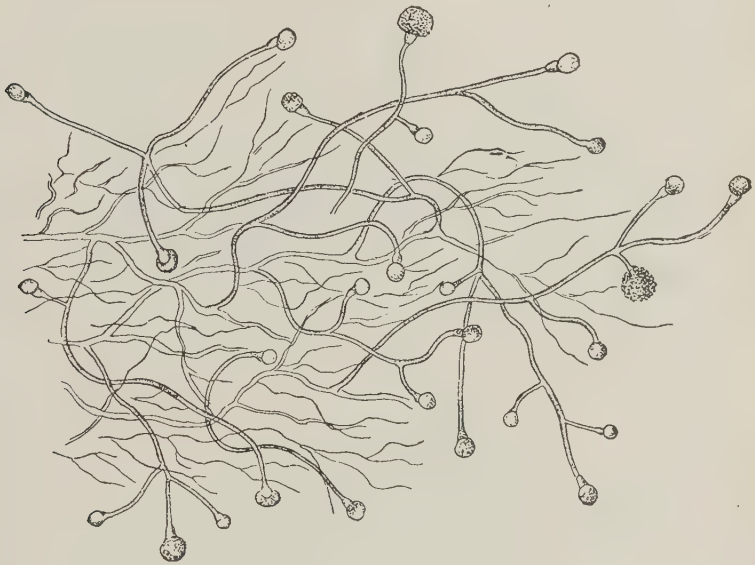


FIG. 172.—Mucor corymbifer, Coh. Mycelium with underlying branched carriers, × 270 diam. (After Lichtheim.)

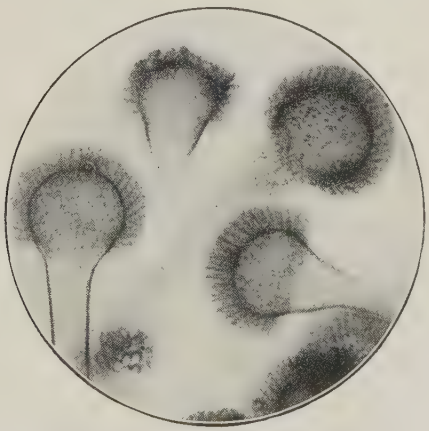


FIG. 173.—Aspergillus fumigatus. Gelatin culture. Spread stained with gentian violet. × 500 diam. (From Itzerott and Niemann.)

animals, of cultivation in artificial media, and of inoculation on man and animals, there are two distinct groups of the fungi causing ringworm

[1] Ann. de Dermat. et de Syph., 1892 and 1893; also Les Teignes, Paris, 1910.

in man—one with small segments (spores) (2μ to 3μ) which is known as *Microsporum*, and one with large segments (spores) (7 μ to 8 μ) which are called *Megalosporon* or Trichophyton. They differ in their mode of growth on artificial media and in their pathological effects on the human skin and its appendages. Among the small-spored varieties or microsporum, is the usual fungus (Microsporum audouini) of Tinea tonsurans of children, especially of those cases which are rebellious to treatment, and their special seat of growth is in the substance of the hair. Megalosporon (Fig. 175) is essentially the fungus of tinea circinata or ringworm of the beard and of the smooth part of the skin; the prognosis as regards treatment is good. One-third of the cases of tinea tonsurans of children are due to megalosporon. The segments or spores of Microsporum audouini are a part of the mycelium. They appear irregularly piled up like zoöglea masses. They may be within the hair (endo-

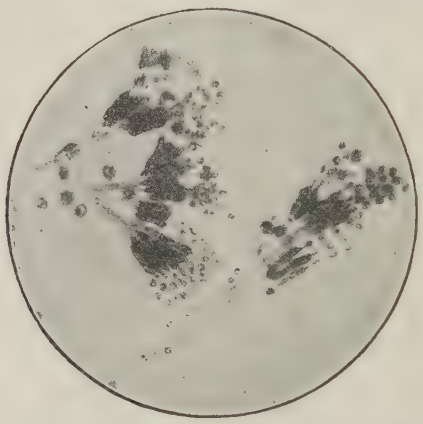


Fig. 174.—Penicillium glaucum. Gelatin culture. Spread stained with gentian violet. 500 : 1. (From Itzerott and Niemann.)

thrix group, Gypseum and Nivcum), or they may form a dense sheath around the hair (ectothrix group). The spores or segments of Megalosporon are always contained in distinct mycelium filaments, which may either be resistant when the hair is broken up or fragile and easily breaking up into spores. The two groups show distinct and constant characters when grown in artificial cultures. The cultures of Microsporum show a downy surface and white color; those of Megalosporon (trichophyton) a powdery surface, with aborescent peripheral rays, and often a yellowish color. Although the morphological appearances, mode of growth, and clinical effects of each group of trichophyton show several similar characters in general, yet there are certain constant minor differences in strains which point to the fact that there are a number of different varieties of trichophyton included under each group. The varieties included under Microsporum are few in number and, with the exception of one which causes the common contagious "herpes" of the horse,

almost entirely human. The varieties of Megalosporon are numerous and fall into several natural subgroups, the members of which resemble one another both from clinical and mycological aspects[1] (Fig. 176).

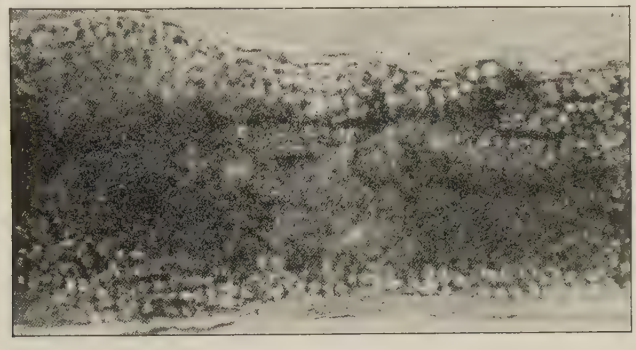


Fig. 175.—Hair riddled with ringworm fungus. Megalosporon variety.

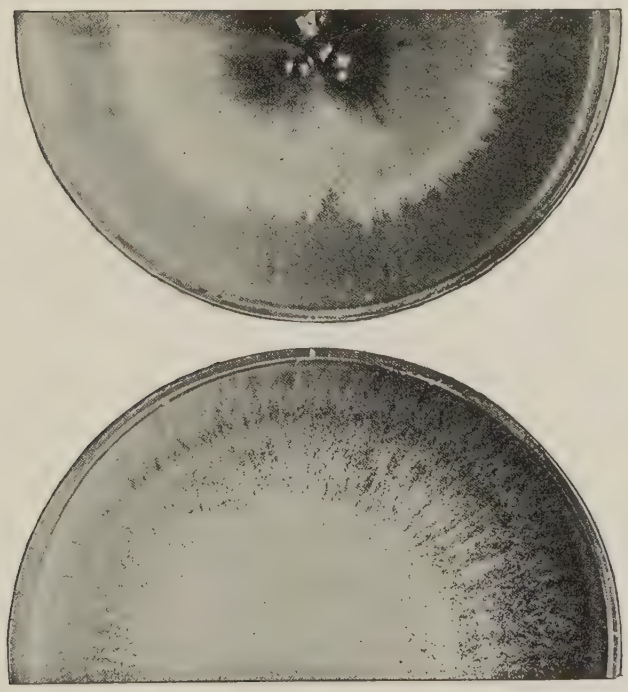


Fig. 176.—These two half-plates show three months' growth on peptone-maltose agar of two megalosporon varieties of the ringworm fungus. Natural size.

[1] For a description of the different pathogenic species of the moulds and yeasts, see Castellani's Fungi and Fungous Disease, Chicago, Am. Med. Assn., 1927, 1928.

Cultures of any strain inoculated into guinea-pigs and other laboratory animals may produce infection.[1]

A number of cases of eczema-like lesions of volar surfaces in man have been shown to be due to a species of ringworm fungus,[2] called *Epidermophyton inguinale* (*Trichophyton cruris*) by Saboraud. White and Greenwood[3] gave a good review of these conditions and their cause. Nails may also be infected with this type of fungus.

Achorion Schoenleinii (Favus).—Favus is due to a fungus discovered by Schoenlein in 1839, and called by Remak Achorion schoenleinii. The disease is communicated by contagion, the fungus being often derived from animals, especially cats, mice, rabbits and fowls; dogs also are subject to it. Buchanan calls attention to the possibility of mouse favus being carried in Australian or other wheat that has been stored for any time and thus made liable to contamination by diseased mice.

The fungus grows much more slowly than the ringworm fungus, and is therefore not so quickly transmitted, but it will surely infect careless, dirty people coming in daily contact with it. Want of cleanliness is a predisposing factor. The fungus seems to find a more favorable soil for its development on the skin of persons in weak health, more in those suffering from phthisis than from other diseases.

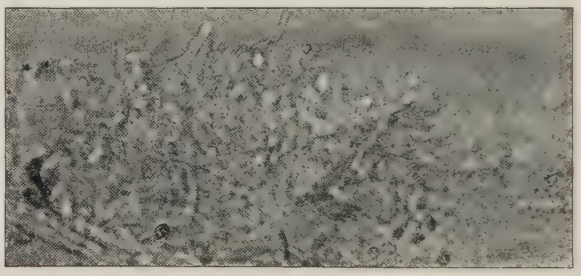


Fig. 177.—A portion of a favus-infected hair; magnified.

Favus produces tissue irritation of a very chronic course and of great resistance to treatment. The spores generally find their way into the hair follicles, where they grow in and about the hair (Fig. 177). It grows in the epidermis, the density of the growth causing pressure on the parts below, thus crushing out the vitality of the hair and giving rise to atrophic scarring. The disease shows a marked preference for the scalp, but no part of the skin is exempt, and even the mucous membranes are likely to be attacked. Kaposi reported a case in which a patient suffering from universal favus died, with symptoms of severe gastro-intestinal irritation, which was found after death to be due to the

[1] For method of examination of these fungi, see Microscopic Methods, p. 91. Sabouraud (Les Teignes, Paris, 1910) should be consulted for details as to cultural varieties.

[2] Ormsby and Mitchell: Jour. Am. Med. Assn., 1916, **67**, 711. Jour. Am. Med. Assn., 1919, **72**, 97.

[3] Jour. Am. Med. Assn., 1921, **77**, 1297.

presence of the favus fungus in the stomach and intestines. Foster[1] reported many cases of favus and ringworm of the nails in immigrants. On the scalp it first appears as a tiny sulphur-yellow disk or scutulum, depressed in the center like a cup and pierced by a hair. This is the characteristic lesion. The cup shape is attributed by Unna to the fact that growth is more vigorous at the sides than at the center. Under the microscope material from a scutulum teased out in a drop or two of 20 per cent. sodium hydrate solution (slightly heated) shows chiefly small, doubly contoured, round or oval spores, single or in chains scattered throughout a dense network of fine threads among which on the edge of the scutulum may be recognized distinct branched hyphæ with swollen ends.

The favus fungus is readily cultivated at the body temperature, less readily at room temperature, in the ordinary culture media, as agar, blood serum, gelatin, bouillon, milk, infusion of malt, eggs, potato. It shows a preference for slightly acid media (p. 136). The growth develops slowly and shows a preference to growth beneath the surface of less solid media. On potato it develops on the surface in characteristic layers. The specific form of growth is that of moss-like projections from a central body (Fig. 178). The color is at first grayish-white, then yellowish. As seen under the microscope, ray-like filaments are developed, which divide into branches. The ends are often swollen or club-shaped, and there are various enlargements along the body of the filament. Several varieties of favus have been discovered.

Recently the roentgen-ray treatment has been recommended for aborting the disease but this is not always practicable. Also specific vaccines have been tried but reports from their use are not satisfactory.

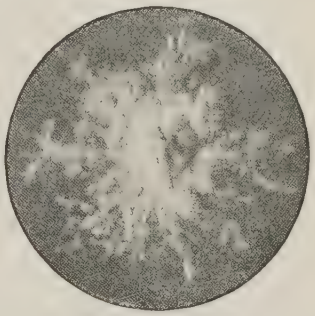


Fig. 178.—Five-month-old colony of favus on peptone-maltose agar; actual size.

Malassezia Furfur (Eichstedt, 1846 and Robin, 1853).—This organism, found in pityriasis (tinea) versicolor, belongs to a group of fungi which, in contrast to the more parasitic fungi, favus and trichophyton, invades only the most superficial layers of the skin and does not give rise to any considerable pathological changes in the skin or hair. Although the vegetative elements of these fungi are much more numerous in the affected portions of the skin than is the case with the more parasitic species, they are not nearly so contagious as the latter.

By preference the organism attacks the chest, abdomen, back, and axillæ, less frequently neck and arms, while exceptionally it attacks also the face. The growth shows itself as scattered spots varying in

[1] Jour. Am. Med. Assn., 1914, **63**, 640.

color from that of cream-coffee to reddish-brown. The spots are readily scraped off and show fine lamellation or scaling. Occasionally the spots are confluent, and sometimes arranged in ring form like Herpes tonsurans.

In spite of their slight contagiousness this is one of the most frequent dermatomycoses. Although it is distributed widely over the earth, it is more frequently observed in southern than in northern countries.

Persons with a tender skin and a disposition to perspire freely are particularly affected by pityriasis versicolor, and this is undoubtedly the only reason why the affection is so frequently observed in consumptives. Women are more frequently attacked than men, while children and old people are rarely affected.

The source of infection is unknown, since the absence of contagion has frequently been demonstrated. It seems likely that the spores of this fungus are so widely distributed that susceptible individuals are easily infected.

The arrangement of the fungus in the scales of the epidermis is characteristic. The short and thick curved hyphæ (7μ to 13μ long and 3μ to 4μ wide) surround large clumps of spores. The spores are coarse, doubly contoured (4μ to 7μ) and round. On staining with Ziehl's solution the spores are seen to contain deeply stained globules lying, in all probability, on the inner surface of the cell membrane. The rest of the protoplasm is but little stained, or not at all. One frequently finds that these globules are also seen free; what their nature is does not appear; they are not found in cultures; the freshly developed spores show only a single globular mass of protoplasm possessing a fine blue luster.

It is claimed that cultures are obtained with difficulty and the growths cannot be transferred for many culture generations.

Sporotricha.—Another of the moulds classed with the *Fungi imperfecti* (family Mucedineæ) has been found to produce disease in man and some of the lower animals. The first variety described was by Schenk[1] in 1898. It was declared one of the sporotricha by F. Smith. In 1900 Hektoen and Perkins[2] described minutely a culture isolated from another case and gave the organism the name Sporotrix (Sporotrichum) schenkii.

Since the disease was made more thoroughly known by the studies of Beurmann and Gougerot[3] and others, many cases have been reported in various parts of the world, and several new species of sporotricha have been described. Beurmann and Gougerot first described the species so far most frequently reported, that is, Sporotrichum beurmanni, which is so similar to Sporotrichum schenkii that they are classed now by most observers as one species. So several of the other species described may prove on further study to be but one species.

Earlier the disease was often mistaken for tuberculosis or syphilis because of the similarity of the lesions which, in general, are slowly

[1] Johns Hopkins Hosp. Bull., 1898, p. 281.
[2] Jour. Exp. Med., 1900, **5**, 77.
[3] Traité des Sporotrichoses, Paris, 1912.

growing granulomata ending in degeneration. When it was found that the disease responds readily to treatment with potassium iodide and other iodine combinations, but not to mercury salts, it became comparatively easy to corroborate the diagnosis. In 1912 Ruediger[1] published a review of the cases reported to date. In the latter part of 1915 Meyer[2] reported that the disease is comparatively common in certain parts of the United States, in domesticated animals, especially in horses, but that human beings do not readily contract the disease from them.

Morphology.—In the pus from the lesions only oval, highly refractile conidia or spores (3μ to 4μ x 1.6μ to 3μ) are seen and these generally sparingly. They may lie within the tissue cells. They are Gram-positive. In cultures these spores grow out into a branching irregularly septate mycelium (the hyphæ about 1μ thick), and the new conidia are formed simply at the sides and ends of the hyphæ without definite fruiting organs. The conidia often occur in whorls. (Plate III, Fig. 5.)

Cultivation.—They grow readily on ordinary media, but better when the reaction is slightly acid. Sabouraud's medium (p. 136) is best. They grow both at room and at incubator (36° C.) temperature. At 20–25° C. growth is seen in about four days, and at 36° C. in about forty-eight hours. Minute, fluffy, snowflake-like colonies appear which slowly become brown or brown-black, and, when in a mass, convoluted. Gougerot found that by letting a drop of the pus flow over a glass slide and keeping in a moist atmosphere, the sprouting of the spores may be followed under the microscope in two or three days. Agglutination and complement fixation have been studied by Widal and Abrami (1908). Their results, which are given in the next paragraphs, await further corroboration.

Agglutination.—Homogeneous emulsions of the spores are made from cultures on Sabouraud's medium six to twelve weeks old. A large amount of the cultures is removed and rubbed until dry in a mortar. To this, drop by drop, is added several cubic centimeters of normal salt solution, while continuing to rub. The suspension is filtered through moistened filter paper. The filtrate contains only free spores which in positive cases agglutinate in from fifteen to sixty minutes in dilutions of 1 to 150 to 1 to 1800, generally 1 to 300 to 1 to 400. Agglutination with the sera of other mould and yeast infections may occur to less extent.

Complement fixation is said to be not specific for the different mould and yeast infections.

Pathogenesis.—Cultures or pus injected subcutaneously or intraperitoneally into lower animals such as mice, rats, and dogs produce granulomatous lesions similar to those found in man.

In man the lesions vary from superficial, non-ulcerative gummata to

[1] Jour. Infect. Dis., 1912, **11**, 193.
[2] Jour. Am. Med. Assn., 1915, **65**, 579.

deep visceral abscesses. Sometimes the diagnosis is difficult to determine. According to Gougerot the following points help in differentiation:

1. Many lesions with general good health.

2. Lesions begin as indolent swellings which gradually become large gummata, on which one or several small ulcers with violet edges may appear. These ulcers discharge a thin yellowish shiny pus, and the center gradually becomes cicatrized with a persistence of the abscess under the skin. Generally there is no adenitis.

3. Microscopic and cultural examinations reveal the organisms. Large quantities of the pus should be inoculated into each culture tube which should not be closed with rubber cap. The dry method should also be tried. Direct smears are not satisfactory.

4. Serum diagnosis may be tried.

5. Animal inoculations give positive results.

6. Treatment with potassium iodide. This produces a cure unless given very late in the disease or unless the mucous membranes are extensively affected. Some cases need treatment for two or three months before improvement is seen. Occasionally death occurs.

PATHOGENIC YEASTS.

The definite biologic relationships of the monilia, the oïdia, the torula and the yeasts, or saccharomycetes, have not yet been determined (see page 42 for classification and general characteristics). The relations of the so-called coccidial organisms have not yet been settled. All we can do is to group them tentatively and wait for further investigations to help us place the pathogenic forms rightly. That we should still call all markedly budding forms blastomycetes and the diseases produced by them blastomycoses seems wiser than to drop these terms and call the infections "torula infections," "oïdiomycosis" or "moniliosis" when we are not yet sure that the organism producing them is a monilia, a torula or an oïdia. But we are sure that these organisms all produce yeast-like forms in abundance which propagate chiefly by budding, and hence may be called blastomycetes.

Stoddard and Cubler[1] (1916) conclude from a study of a culture obtained from a case of infection of the nervous system that the microörganism is a torula because it reproduces only by budding, it forms no mycelium and it produces no gaseous fermentation; and they further conclude that because of apparently similar lesions in other cases reported the same germs might have been the cause in each.

Vuillemin, Castellani and others have called the fermenting type of yeast-like forms found in man, Cryptococcus hominis; the non-fermenting type described by Gilchrist, Vuillemin has called Cryptococcus gilchristi, and Castellani, Cryptococcus dermatitides.

The pathogenic blastomycetes reported may be briefly summarized as follows:

Saccharomyces busse is the name given to a yeast isolated in 1894 by O. Busse from an abscess in the tibia of a thirty-one-year-old woman who died thirteen months after the first symptoms appeared. The autopsy showed numerous

[1] Torula Infection in Man. Monograph 6 Rockefeller Institute, Jour. Med. Res., 1916. New York.

broken-down nodules on several of the bones, and in the lungs, spleen, and kidneys. The yeast was cultivated from all these foci.

Saccharomyces tumefaciens was isolated in 1895 by Curtis. The patient was a young man showing multiple tumors on the lips and neck having the gross appearance of softened myxosarcomata. This yeast is pathogenic for rats, mice, and dogs, only slightly so for rabbits, and not at all for guinea-pigs.

Various similar cases have since been described, a number of them becoming generalized and ending fatally. In generalized blastomycosis the lung seems frequently to be the seat of primary infection. Several cases of mixed infections have been reported as due to yeast and tubercle bacilli. Samplice[1] states that the lungs of tuberculous cattle often contain blastomycetes and other fungi and that the blastomycetes are indistinguishable from those isolated by him from human tumors. They seem to render the tuberculous infection more virulent.

The cases described first by Rixford and Gilchrist as coccidiosis due to "Coccidioides imitis"—thought to be a protozoön—may be classed here, since Ophüls, Oliver, and others have shown that the "coccidium" forms hyphæ and elliptical forms on culture. Cummins and Sander[2] showed that cultures from fresh tissue grow slowly (appearing in from seven to twelve days in primary cultures) but with ease on the usual laboratory media. The growth is more mould-like than yeast-like, except on potato, when yeast-like cells are produced, though no budding has been seen. Dogs, rabbits, and guinea-pigs are susceptible to the fungus and show lesions similar to those in human beings. The disease has been called coccidioidal granuloma. Zinsser[3] isolated an organism similar to those described by Ophüls and Oliver from an abscess of the back. Brown and Cummins[4] state, after an extensive study of "coccidiodal" disease, that the species causing this group of cases is distinct from that caused by the definitely budding type of yeast.

A typical case of systemic blastomycosis, reported by Fontaine, Hasse, and Mitchell,[5] is accompanied by very good illustrations of tissue sections. Fig. 179 shows the characteristic microscopic appearance of the lung lesion.

Several cases of ophthalmia have been described as due to yeasts. Recently Jackson[6] and others have added to the reports of cases of eye infection by yeasts.

Neal,[7] in our laboratory, had an interesting case of meningitis, due to a budding form which was classed as a torula.

Buschke isolated a yeast from a cervical discharge in which no gonococci were present. The yeast was pathogenic for guinea-pigs.

In 1895 Tokishige reported an epidemic quite common among horses in Japan, known as "Japanese worm," "benign worm," or "pseudoworm," which is caused by one of the saccharomyces. This disease begins in the skin in the form of hard, painless nodules from the size of a pea to that of a walnut. These break down and give rise to gradually extending ulcers. Pure cultures of the saccharomyces are pathogenic chiefly for horses, not for rabbits, guinea-pigs, or hogs. In the districts where the disease prevails among horses it is also frequently seen in cattle.

Shortly after Tokishige's publication a similar disease occurring in horses in Italy and southern France was identified as being caused by saccharomyces. Cultures of this yeast, however, differ somewhat from that obtained in Japan, so that Busse is inclined to regard the two as two different species of blastomycetes.

Kartulis, in Alexandria, described about a hundred cases of a skin affection occurring in the gluteal regions of men and characterized by an elongated finger-like swelling, which breaks and emits a purulent discharge, forming an unhealed sinus. In the discharge and surrounding tissues are numerous blastomycetes

[1] Annals d'Igiene. 1918, **28**, 41.
[2] Jour. Med. Res., 1916, **35**, 243.
[3] Proc. New York Path. Soc., 1907.
[4] Arch. Int. Med., 1915, **15**, 608 (with bibliography).
[5] Ibid., 1909, **4**, 101.
[6] Jour. Am. Med. Assn., 1915, **65**, 23.
[7] Arch. Nurol. and Psych., 1925, **13**, 174.

which Kartulis after cultivation and study considered a variety of the ordinary fermenting yeast (Saccharomyces cerebisiæ Hansen). The cases were cured by excising the growth.

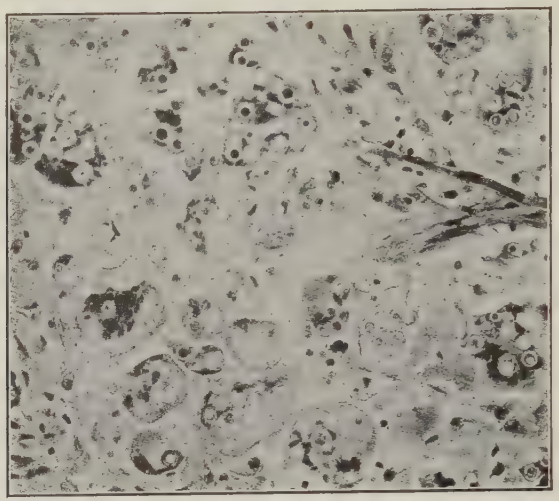


Fig. 179.—Section of lung. × 150; blastomycetes in large syncytial cell masses. (From Fontaine, Hasse, and Mitchell.)

Kessler[1] reported a skin lesion in an infant (Fig. 180) probably due to a similar blastomycete, since the lesions healed after treatment with potassium iodide. The description of the yeast isolated is too incomplete to identify it.

Fig. 180.—Blastomycosis in infant aged eight months, showing lesion on left cheek. (Kessler.)

Some years ago the attempt was made to connect the development of cancerous growth with blastomycetes. This was due in a measure to a certain similarity between the yeasts and the cell inclusions or so-called "parasites" of cancer, and

[1] Jour. Am. Med. Assn., 1907, **49**, 550.

further, to the fact that when yeasts are injected into the animal body tumor-like nodules are often developed at the site of inoculation and in the internal organs. But these nodules are not tumors in the pathological sense of the term, but merely masses of blastomycetes mixed to a very variable degree with inflammatory tissue proliferations.

Thrush (*Oöspora, Oïdium Albicans, Monilia Albicans*) (Fig. 181).—Thrush, as is well known, occurs most frequently in the oral mucous membrane of infants during the early weeks of life. It may also be found as a mycosis in the vagina, especially of pregnant women. In rare cases the disease attacks adults, and especially those whose system has been undermined by other diseases, such as diabetes, typhoid, etc. A few cases are recorded in literature in which this fungus has given rise to constitutional disease. In these cases autopsy has shown abscesses in various parts of the body, such as the lungs, spleen, kidney and brain.

Fig. 181.—Inflammation of cornea by thrush (Oïdium albicans), *g*, conidia; *e*, pus cells. (From Plaut, in Kolle and Wassermann.)

The tropical disease called *sprue* characterized by diarrhea, progressive wasting and anemia, was said by Baker to be due to Monilia albicans. Ashford[1] thinks this disease is caused by a distinct species of monilia, Monilia psilosis. This is probably identical with Castellani's Monilia enterica. Castellani has obtained a number of different varieties of monilia from the feces in tropical sprue.[2] Others have also obtained strains that vary in some particulars (Famulener and Hewett,[3] Bastedo[4]).

In the lesions of the disease as well as in cultures this type of fungus appears as both a yeast and a mycelium. It thus seems to stand between the true moulds and the yeasts. The yeast cells are oval in form, about 5 μ to 6 μ long and 4 μ wide, and can in no way be distinguished from other cells either by their appearance or their method of propagation. The threads of the mycelium vary markedly in length and thickness, and show all intermediate forms between a typical

[1] Etiology of Sprue, Am. Jour. Med. Sci., 1917, **154**, 157.
[2] See Castellani's 1928 report already quoted.
[3] Proc. New York Path. Soc., 1923, **23**, 159.
[4] Jour. Am. Med. Assn., 1923, **81**, 2102.

and a budding mycelium. Cultures are easily obtained on artificial media especially on those favorable for yeasts (p. 136).

Michael[1] reported favorable results in tropical sprue by treating with a vaccine made from Monilia psilosis strains.

Thrush is not much influenced by acids or alkalis. On the other hand, it is very susceptible to the common disinfectants, especially salicylic acid, corrosive sublimate, phenol, iodine, etc. This fact is made use of in local treatment.

The important part played by yeasts in certain industries is treated in Part III.

[1] Monilia Vaccine in Sprue, Jour. Inf. Dis., 1918, **22**, 53.

CHAPTER XXXVIII.

FILTRABLE VIRUSES. DISEASES OF UNKNOWN ETIOLOGY.

FILTRABLE VIRUSES.

There exists a class of infectious diseases from which it has been quite impossible up to the present time to demonstrate visibly any individual microörganisms, although infective material from such diseases may, with certain precautions, be passed through stone-filters of varying degrees of porosity (for types of filters, see Chapter IV); the filtrates will contain the virus and be capable of reproducing the disease with all its characteristics when inoculated into a susceptible animal. Examined microscopically, even with the highest powers, the filtrate is limpid, and, except in a few diseases which will be described in detail later on, not a sign of characteristic particulate matter can be seen. Such a filtrate therefore contains either ultramicroscopic organisms or small organisms with refraction and staining powers so faint that they cannot be demonstrated by our present methods.

Certain precautions must be observed in such filtrations. In the first place the filter must be shown by actual test to be free from infection. Any and all of the test organisms must be absolutely retained and none pass into the filtrate. A few minute species of germs have been found to pass certain grades of filters, especially under pressure. Thus the bacillus of guinea-pig pneumonia, which is 0.5μ x 0.7μ, passes Berkefeld V (Wherry); a spirillum isolated by von Esmarch passes, according to him, the Berkefelds, Chamberland F, and other filters; a minute water flagellate was found by Borrel to pass through the coarser filters. Wolbach and Binger and others have shown that several spirochetes, pathogens as well as saprophytes, pass through Berkefelds V, N and W, by pressure and by suction. The Bacterium pneumosintes is reported by Olitzky and Gates to be a filter passer. It is seen, therefore, that certain known organisms within the limits of visibility may pass even some of the finer filters. The filtration must be completed within a moderate time, because even bacteria as large as the typhoid bacillus may, in media containing a certain amount of albuminous material, grow, in time, through a filter. The material to be filtered should, furthermore, be greatly diluted and first filtered through filter paper or other coarse filter or be lightly centrifugalized in order to avoid the clogging action of extraneous material.

When a virus fails to pass through a filter the following factors may be responsible:

1. Particulate size of virus is greater than size of filter.
2. Adsorption of virus by walls of filter directly or on particulate substances in medium which fail to pass.

Excluding size of particles as a factor, substances to pass through electro-negative filters with minimal loss may be adjusted to the alkaline side of their iso-electric point with due regard for the effect of the pH used on the active principle.[1]

If after all the proper precautions have been taken the filtrate is pathogenic, it must be shown that the symptoms are due to a micro-organism and not to a toxin. This may be decided by inoculating a series of animals successively with the filtrate obtained from a previously so inoculated animal.

From our present knowledge, filtrable agents may be divided into two groups: (1) those which have not yet been morphologically demonstrated (ultramicroscopic?); (2) those which are shown to be within the limits of visibility. A third group may be made of the diseases produced by viruses of questionable filtrability.

A good résumé of the whole subject is given in the book edited by Rivers already mentioned.

GROUP I.—DISEASES PRODUCED BY FILTRABLE AGENTS OF UNKNOWN MORPHOLOGY.

Foot-and-mouth Disease.—This is probably the first disease shown to be produced by a filtrable agent. It is a highly infectious disease of cattle. Other cloven-footed animals may also be attacked. It occurs seldom in man and then among people handling infected cattle or drinking the milk of infected animals. The disease in cattle is characterized by the appearance of vesicles in the mouth and around the coronet of the foot as well as between the toes.[2] Löffler and Frosch[3] in 1898 discovered that after diluting the contents of an unbroken vesicle with twenty to forty times its volume of water and passing the resulting fluid through a Berkefeld filter, the filtrate contains the virus. This virus remains infectious for some time. One attack of the disease usually produces a certain amount of immunity. Löffler produced a serum which has immunizing properties. The report of the foot and mouth commission[4], published in 1928, gives a detailed account of the recent investigations of this disease.

Mosaic Disease of Tobacco.—This is the second disease in which a filtrable virus was demonstrated. The disease causes the young tobacco leaves to become devoid of chlorophyll in spots which enlarge, and turn brown, and the underlying tissue becomes necrotic. Beijerinck, in 1899, showed that the filtrate from a porcelain filter promptly reproduced the disease on tobacco leaves.

Cattle Plague (Rinderpest).—This fatal European and African disease of cattle is characterized by inflammation of the intestinal

[1] See Mudd's review in Filterable Viruses, Ed. by Rivers, Baltimore, Williams & Wilkins, 1928.

[2] Mohler: Farmers' Bull., 666, U. S. Dept. Agric., 1915.

[3] Centralbl. f. Bakt., 1898, **23**, 377.

[4] Olitzky, Traum and Schoening. Technical Bulletin No. 76, U. S. Dept. Agricul., June, 1928.

mucous membrane. The blood is infectious and filtrates of it through Berkefeld and Chamberland F (Nicolle and Adil-Bey, 1902) produce the disease. No organism can be seen. Immunity follows one attack. It can also be produced by a subcutaneous inoculation of bile from an infected animal.

Yellow Fever.—The undiluted serum from cases of this disease was shown by the American commission (1901) and others (see Chapter XLVI) to pass the Berkefeld and the Chamberland B filters as clear filtrates, and in this form to be infectious; therefore some forms at least of the specific organism are ultramicroscopic.

Rabies.—No bacteria have been discovered that have been proven to be related to etiology. The probability of the Negri bodies being microbes and the exciting factor is considered in Chapter XLVI. The virus of rabies has been shown to be filtrable (Remlinger, 1903). Poor and Steinhardt[1] (1913) showed that gland virus is more filtrable than that from the brain. It may pass through the coarser Chamberland filters.

Trench Fever.—Soon after the beginning of the World War there began to appear among the troops in France, especially those in trenches, many cases of acute fever characterized by dizziness, headache, backache and pains in the legs. The temperature curve shows an intermission on the third or fourth day, followed after a day or two by a second rise and a fall about the eighth day from beginning. There may be from one to several relapses at fairly regular intervals. There is no mortality. Many studies have been made to show that the disease is an infectious entity. McNee, Renshaw and Brunt[2] proved that the whole blood could transmit the disease to healthy soldiers by intravenous or intramuscular inoculation. A few experiments by Davies and Wildon[3] showed that lice probably transmitted the disease. This was proved by members of the Medical Research Committee of the American Red Cross headed by Strong.[4] They summed up their work as follows:

1. Trench fever is a specific infectious disease.
2. The organism causing the disease is a resistant filtrable virus.
3. The virus causing trench fever is present particularly in the plasma of the blood. It is also present in washed red blood cells, in urine and in sputum.
4. The disease is transmitted naturally by the louse Pediculus humanus, Linn., var. corpus, and this is the important and common means of transmission. The louse may transmit the disease by its bite alone, the usual manner of infection, or the disease may be produced artificially by scarifying the skin and rubbing in a small amount of the infected louse excrement.
5. A man may be entirely free from lice at the time he develops

[1] Jour. Inf. Dis., 1913, **12**, 202.
[2] British Med. Jour., 1916, **1**, 225. [3] Jour. Roy. Army Med. Corps, 1918, **30**, 92.
[4] Trench Fever Report of Commission Medical Research Committee, American Red Cross, 1918, University Press.

trench fever, the louse that infected him having left him some time previously, and the louse need only remain upon the individual for a short period of time in order to infect him.

6. Since urine and sputum are sometimes infective they should be sterilized.

7. To prevent trench fever, or limit its spread and thus save manpower, greater efforts should be made to check infestation with lice.

Spirochetes and several kinds of minute organisms have been respectively described as the cause of trench fever but further studies are awaited for proof.

The final report of the English committee was given by Bruce.[1]

Hog Cholera.—de Schweinitz, Dorset, Bolton and McBryde demonstrated in 1905 that the blood of hogs suffering from hog cholera contains a filtrable virus which is capable of producing the disease on inoculation into healthy hogs. This virus passes Chamberland B and F filters. It leaves the body in the urine and probably enters another animal through contaminated food. King, Baeslack and Hoffman[2] found in 1913 a short motile spirochete (Spirocheta suis) in the blood in a series of cases of hog cholera. They think this organism may be a stage of the specific organism which may produce filtrable granules.

This disease is a very fatal and contagious disease of swine characterized by fever and ulcerative enteritis. Immunity follows one attack. An immune serum is produced. Animals are also immunized by a mixture of infected blood and immune serum (sensitized virus). The hog cholera bacillus was earlier supposed to be the cause of this disease.

South African Horse Sickness.—This is a warm-weather disease, more common in animals that do not pass the night under cover. The horses are uneasy, have difficulty in breathing, and a reddish froth exudes from their mouths. The temperature rises in the daytime, but has a tendency to drop at night. In severe cases an edematous swelling of the head and neck may appear. MacFadyen succeeded in passing blood serum of a diseased horse (diluted) through the Berkefelds and a Chamberland F, not through a Chamberland B filter.

Chicken Sarcoma.—In 1910 Rous[3] discovered a spindle-celled tumor in chickens, which he has reproduced in other chickens by transplantation, by inoculation of dried tumor tissue, and by the inoculation of Berkefeld filtrates from emulsions of tumor cells.

Novy's Rat Virus.—Novy reported finding an extremely virulent filtrable virus from a disease of rats, which passes all filters.

Several diseases formerly placed among those of questionable filtrability must be accepted at present as filtrable.

Smallpox and Related Diseases.—The details of the disease smallpox are considered in a later chapter. In 1908 Casagrandi reported that

[1] Jour. Hyg., 1921, **20**, 258.
[2] Jour. Inf. Dis., 1913, **12**, 39 and 206.
[3] Rous and Murphy: Jour. Exp. Med., 1912, **15**, 270.

the virus was filtrable under pressure through coarser Berkefeld filters. Vaccine virus had already been reported as filtrable by some investigators, and as non-filtrable by others, but later experiments as those of Ward[1] show that by observing certain conditions about 5 per cent. of the virus will pass a Berkefeld V filter.

Dengue.—Ashburn and Craig report having reproduced dengue in susceptible individuals by a procedure similar to that employed in yellow fever. The virus they showed passes a Berkefeld filter. The intermediary host in natural infection is aëdes ægypti. Craig[2] thinks the disease is due to a spirochete. Siler, Hall and Hitchens[3] gave a comprehensive review of dengue. They were unable to demonstrate spirochetes as the cause.

Measles.—This very definite infectious exanthematous disease still remains among those of unknown etiology. Cell inclusions, bacilli and cocci in turn have been reported as having an etiological relationship. The reports have not been corroborated. In 1905 Hektoen[4] produced measles in two human cases by the inoculation of blood drawn from an infected case at an early stage of the disease. Anderson and Goldberger,[5] in 1911, inoculated monkeys with measles blood and demonstrated the virus in the blood of the inoculated animals and in the secretion of the upper air passages. They also demonstrated the period of infectivity of the blood. They reported positive results with Berkefeld filtrates. Tunnicliff[6] reported finding a small anaërobic coccus in the blood of 42 out of 50 cases of measles. She states that there seems to be a specific increase of opsonins, complement-fixing bodies and agglutinins for this coccus early in measles patients. Blake[7] repeated the work on measles and produced rather typical symptoms in a small percentage of monkeys with filtered and unfiltered virus. He thus corroborated Anderson and Goldberger. Nevin and Bittman,[8] and Grund,[9] also showed that monkeys and rabbits are susceptible to the filtered and unfiltered virus. Duval and D'Aunoy[10] repeated the work in rabbits with positive results. More recently Tunnicliff[11] reported further studies on her streptococcus, including the obtaining from immunized goats of a serum having protective properties. Ferry and Fisher[12] reported finding a similar coccus, the toxin of which stimulated the production of an antitoxin. We[13] were not able to corroborate their results. Neither have we been able to establish Degkwitz' claim[14] that he can not only grow a virus but also obtain an antiserum.

[1] Jour. Exp. Med. 1929, **50**, 31.
[2] Jour. Am. Med. Assn., 1920, **75**, 1171.
[3] Siler, Hall and Hitchins: Dengue, Manila Bureau of Printing, 1926.
[4] Jour. Inf Dis., 1905, **2**, 238.
[5] Public Health Reports, 1911, 1912 and 1913; Jour. Am. Med. Assn., 1911, **58**, 113.
[6] Jour. Am. Med. Assn., 1917, **68**, 1029.
[7] Jour. Exp. Med., 1921, **33**, 413.
[8] Jour. Inf. Dis., 1921, **29**, 429.
[9] Ibid., 1922, **30**, 86.
[10] Jour. Exp. Med., 1922, **36**, 231.
[11] Jour. Am. Med. Assn., 1926, **87**, 2139, and 1927, **88**, 1028.
[12] Jour. Am. Med. Assn., 1926, **86**, 932.
[13] Park, Williams and Wilson: Am. Jour. Pub. Health, 1927, **17**, 460.
[14] Münch. med. Wchnschr., 1926, vol. **5**.

Convalescent Serum.—We[1] were able to demonstrate clearly the marked value of measles convalescent serum in preventing or modifying measles. This has value even when taken from an adult who had measles in childhood. The dose then is five times as large.

Warts.—Wile and Kingery[2] reported obtaining a filtrable virus from common warts which will produce warts on humans.

Canine Distemper.—The studies of Laidlaw and Dunkin[3] present strong evidence that canine distemper is caused by a filtrable virus. They worked out the course and immunity of the disease in ferrets and tested their results in dogs bred under an elaborate aseptic technic. They state that a vaccine made with the virus produced definite and lasting immunity.

GROUP II.—DISEASES PRODUCED BY FILTRABLE VIRUSES THE AGENTS OF WHICH ARE VISIBLE OR PROBABLY VISIBLE.

Contagious Pleuropneumonia of Cattle.—This malady affects cattle but not other species. Typically, there is an inflammation of the lungs and the pleura which is invasive and causes necrosis of the diseased parts. Nocard and Roux succeeded in cultivating the organism in collodion sacs placed in the peritoneal cavity of rabbits, using a mixture of serum and bouillon. After two weeks a very faint turbidity appeared in the sacs; coincidently the fluid became infective. Nocard and Roux described the organisms as minute spheres and spirals just within the limits of visibility. They showed (1899) that the organisms passed the Berkefelds and a Chamberland F, but not a Chamberland B filter. Immunity is produced by a single attack. It has also been produced artificially by the inoculation of cultures or virulent exudates.

Epidemic Poliomyelitis.—This is a disease which affects chiefly the central nervous system. It occurs usually in children, and appears sporadically and in epidemics in many countries. In New York, in 1907, there was an epidemic of over 2000 cases and in Texas, in 1912, there was a large outbreak. During the summer of 1916 one of the largest epidemics known occurred in New York City and the surrounding country.[4] Since then several small scattered outbreaks have occurred. In Nature, the virus enters probably through the upper air passages. The chief symptoms of the disease are fever, with or without sore throat, hypersensitiveness followed by paresis and paralysis. The mortality is low. There is usually permanent injury to parts of the motor areas of the nervous system, with resulting deformity. The principal microscopic changes are a marked exudation of polynuclear leukocytes into the lymph spaces and the cerebrospinal fluid. The changes are usually specially marked in the anterior commissure and the cornea of the cervical and lumbar regions of the cord, but the whole nervous system is

[1] Park and Freeman: Jour. Am. Med. Assn., 1926, **87**, 556.
[2] Jour. Am. Med. Assn., 1921, **76**, 440. [3] Brit. Med. Jour., 1928, **2**, 1100.
[4] Monograph Series No. 16, New York City Health Department, 1916; Public Health Bull. No. 91, U. S. Pub. Health Service, 1918.

more or less affected. The disease seems to be closely related to certain diseases manifested by acute encephalitis and bulbar paralysis, *e. g.*, Landry's paralysis.

Landsteiner and Popper (1909) reported the transmission of acute poliomyelitis to apes. They inoculated the spinal cord intraperitoneally and produced typical symptoms and lesions, but did not succeed in transmitting from ape to ape, probably because they used a mild virus. Flexner (1909) and Lewis[1] transmitted the disease from monkey to monkey by means of intracerebral inoculations. Landsteiner and Levaditi[2] (1909) also transmitted it from monkey to monkey. They found that the virus lives some time outside of the body; that the degenerated nerve cells are taken up by phagocytes, and there is an analogy between the lesions of poliomyelitis and those produced by rabies. They demonstrated that the virus is filtrable. Leiner and Wiesner transmitted the disease from monkey to monkey, found young animals more susceptible than older ones, and spinal fluids, blood, and spleen negative. Flexner transmitted the disease by inoculating into the blood or peritoneal cavity, also by the subcutaneous method, and independently found the virus to be filtrable. Landsteiner and Levaditi found the virus in the salivary glands and suggested the saliva, moist or dry, as a source of contagion.

Soon after this (1911) Noguchi and Flexner[3] announced that they had obtained cultures. In their media a sediment gradually forms. Giemsa's stain shows the presence of minute globoid bodies (0.15 μ to 0.3 μ diam.) in pairs, short chains, and masses. The authors considered these bodies the cause of the disease. Levaditi stated that he could not obtain the results of Noguchi, but that he did get evidence of growth by the living tissue method. Rosenow[4] states that he found an aërobic streptococcus which shows in early culture generations specific pathogenicity for the central nervous system.

During the epidemic of 1916 we also studied the etiology of this disease, but we were not able to corroborate any of these investigators.

Rabbits and possibly guinea-pigs have also been found by some to be somewhat susceptible to the virus (Krause and Meinicke,[5] Marks,[6] Rosenau[7]). The present opinion is that the disease is due to an undiscoverable filtrable virus.

Immunity.—One attack seems to give certain immunity. Flexner and Amoss[8] found that, experimentally, the virus inoculated into the blood is capable of being neutralized by intraspinous injections of immune serum. A series of vaccinations similar to that given as a prophylactic against rabies may produce immunity.[9] Since then we have

[1] Jour. Am. Med. Assn., 1909, **53**, 1913 and 2095, **41**, 1639; Jour. Exp. Med., 1910, **12**, 227.
[2] Compt. rend. Soc. de biol., 1909, **47**, 592 and 787.
[3] Jour. Exp. Med., 1913, **18**, 461.
[4] Jour. Med. Res., 1917, **36**, 175, and Jour. Inf. Dis., 1918, **22**, 281.
[5] Deutsch. med. Wchnschr., 1909, **35**, 1825.
[6] Jour. Exp. Med., 1911, **14**, 116.
[7] Ibid., 1916, **23**, 461.
[8] Ibid., 1913 and 1914.
[9] Abramson: Jour. Am. Med. Assn., 1918, **70**, 1142.

produced a potent antiserum by giving intravenous injections of suspensions of the living virus to horses.

Treatment.—Serum from convalescent human cases has been used in the treatment of early cases with apparent success. Some good observers doubt its value. If a case is diagnosed before paralysis has developed an intraspinal injection as well as an intravenous is given. The following day a second intraspinous injection is given.

We have developed a potent serum by injecting a horse repeatedly in the vein with an emulsion of the brain or spinal cord of a monkey which had died of poliomyelitis. This has been refined and concentrated by Banzhaf. The globulin solution protected a monkey when given on the day after the cerebral inoculation.

Epidemic Encephalitis.[1]—During 1918 a series of cases occurred in England and France which were first thought by some to be atypical cases of poliomyelitis. That doubt prevailed as to their nature was shown by the various names applied to the condition—acute encephalitis, infective ophthalmoplegia, poliomyelitis, botulism, lethargic encephalitis, etc. The majority of observers now think that the disease is a clinical entity similar to the one described by Enconomo in Vienna in 1917, and to cases described as following the epidemic of influenza in 1890 especially in northern Italy, where it received the name of Nona. The disease is characterized by three cardinal signs: lethargy, asthenia and cranial nerve palsies. Levaditi[2] and Harvier believe they have reproduced the disease in monkeys and rabbits and have shown that the virus is filtrable. The virus has not been demonstrated in sections unless the "neurobodies" found in brain sections represent the virus. Some, at least, of the so-called encephalitis viruses said to produce lesions in rabbits are probably those of spontaneous encephalitis of the rabbit. The sections in such cases should show the Encephalitozoön cuniculi. Neal[3] has given a good review of the present status of the etiology of epidemic encephalitis.

Postvaccinal encephalitis is supposed to be due to another virus which probably exists in certain countries as a harmless commensal unless incited to action by the presence of certain other infectious principles.

Relapsing Fever.—Todd and Wolbach have found (1914) that Spirocheta duttoni may be forced through even the finer grades of Berkefeld filters by a pressure of from 50 to 90 pounds to the square inch. For filtrability of other spirochetes see page 609.

Mumps.—Comparatively few studies have been made of this infectious disease, probably because of its low mortality. By puncturing the parotid gland, Laveran and Catrin obtained a diplococcus which stains easily, is Gram-negative, and grows on ordinary culture media. No satisfactory specific studies have been made of this organism.

[1] Neal: Monthly Bull. Health Dept., New York City, 1919, **9**, 69; also Jour. Am. Med. Assn., 1919, **72**, 714; Arch. Neurol. and Psychiat., 1919, **2**, 271; Arch. Pediat., 1921, **37**, 321; Jour. Am. Med. Assn., 1921, **77**, 121.

[2] The Journal of State Medicine, **32**, No. 6, 201 and 251.

[3] Jour. Am. Med. Assn., 1928, **91**, 231.

Wollstein[1] showed that a filtrable virus can be obtained from the secretions from mumps cases which causes half-grown cats to react specifically in the parotid and testes, and that the virus is detected most readily during the first three days of the disease and not on the ninth day. One attack in humans usually gives a lasting immunity.

Hess,[2] in 1915, used serum from recovered cases of mumps in children's homes as a protective measure, with the result that none of the 17 so inoculated came down with the disease on exposure.

GROUP III.—DISEASES PRODUCED BY VIRUSES OF QUESTIONABLE FILTRABILITY.

Rubella.—Tunnicliff[3] found a coccus in a series of rubella cases for which she reported specific antibodies.

Trachoma.—This condition has already been considered in a previous chapter. Bertarelli reported that he was able to produce a specific filtrate. Nicolle[4] and Noguchi[5] also reported positive results with filtrates. The descriptions of the disease, however, are too vague to allow an opinion as to the truth of these reports.

Noguchi[6] described a small bacillus as the possible cause of "trachoma" in certain American Indians.

Typhus Fever.—The occurrence of this infectious disease in epidemic form had disappeared from civilized lands until the World War broke out. The fact that the body louse may transmit the disease helps explain why it has always been classed as a filth disease. It still occurs endemically in parts of Europe and North and South America. In Mexico it occurs in epidemics where it is known as Tabardillo. In New York it occurs occasionally in mild form under the name of Brill's disease. Brill thought it was a new disease, but Anderson and Goldberger[7] showed that typhus fever and Brill's disease are the same. Nicolle and Krumwiede[8] observed 4 cases which clinically verified these findings. The disease is characterized by high temperature and a petechial rash.

Nicolle (1909) showed that the old world typhus can be transmitted to the chimpanzee and from this to the macacus with typical eruption in each case. He also showed that the disease is transmitted by the ordinary body louse (Pediculus vestimenti) seven days after an infected meal, and not after the tenth day. Anderson and Goldberger (1909) were the first to transmit typhus fever of Mexico (tabardillo) to monkeys. They were able to transmit directly from human beings to the macacus and capuchin and from monkey to monkey. Ricketts and Wilder (1910) also found that the macacus was directly susceptible to the disease. They based their diagnosis chiefly upon a rather indefinite fever

[1] Jour. Exp. Med., 1916, **23**, 353, and 1918, **28**, 377.
[2] Am. Jour. Dis. Child., 1915, **10**, 99.
[3] Jour. Inf. Dis., 1918, **22**, 462.
[4] Compt. rend. Acad. Sc., 1909 and 1911; Ann. de l'Inst. Past., 1910, 1911 and 1912
[5] Jour. Am. Med. Assn., 1918, **71**, 1201.
[6] Ibid., 1927, **89**, 739.
[7] Public Health Rep., 1910, 1912 and 1913; Jour. Am. Med. Assn., 1911, **57**, 113.
[8] Jour. Am. Med. Assn., 1912, **59**, 521.

and in most cases somewhat distinct symptoms of illness. They also found that the monkey may pass through an attack of typhus so mild that it cannot be recognized clinically, but results in immunity. The immunity test is a reliable proof of the previous occurrence or non-occurrence of typhus at least within a period of one month. They found that typhus was transmitted to the monkey by the bite of the louse. They further state that in stained preparations of blood of patients taken from the seventh to the twelfth day of the disease they invariably found a few short bacilli similar to those which belong to the hemorrhagic septicemia group. In moist preparations they saw similar forms in all cases. No motility was observed. No cultures could be obtained. They examined the dejecta of many lice and found similar bodies, since called Rickettsia, in large numbers in infected lice and occasionally in non-infected lice. Pediculus capitis may also transmit the disease. Ricketts and Wilder and Anderson and Goldberger showed that the virus could also be transmitted to guinea-pigs. Their chief reaction is a rise of temperature of 2 to 3 ° on the sixth to eighth day after inoculations.

First attempts at filtration by Ricketts and Wilder,[1] Anderson and Goldberger[2] and Nicolle,[3] Connor and Wilder gave negative results. Olitzky[4] went over this work and added experiments of his own which seemed to show that the virus is non-filtrable. Nicolle and co-workers later reported some positive results.

As a result of the World War there were several severe epidemics of typhus. The Serbian epidemic was particularly severe and the disease was studied by several groups of investigators, among them the Red Cross Committee headed by Strong.[5] During this epidemic about 150,000 deaths were reported within six months. These investigations brought out the fact that Rickettsia bodies are found in lice in connection with a number of diseases. Wolbach, Todd and Palfry[6] in their extensive studies of the disease in Poland gave a tabulation of the various Rickettsia so far reported. They consider the Rickettsia of typhus fever distinctly different from the others. They have also given beautiful demonstrations of intracellular granules in endothelial cells and have reported an increase of them by cultural methods.[7] Reviews of the subject Rickettsiæ have been given among others by Wolbach[8] and Cowdry.[9]

Agglutinins in Blood of Typhus Fever Patients.—Weil-Felix Reaction.—Wilson[10] called attention to the presence in the serum of typhus fever

[1] Jour. Am. Med. Assn., 1910, **60**, 309.
[2] Public Health Rep., 1910, 1912 and 1913; Jour. Am. Med. Assn., 1911, **57**, 173.
[3] Compt. rend. Acad. Sci., 1909 and 1911; Ann. de l'Inst. Past., 1910, 1911 and 1912.
[4] Jour. Inf. Dis., 1917, **20**, 349.
[5] Typhus Fever, etc., Report Red Cross Serbian Epidemic, Harvard Univ. Press, 1920.
[6] Main Rep. Typhus Res. Com. Red Cross Soc., Poland, Harvard Univ. Press, 1922.
[7] Wolbach and Schlesinger: Jour. Med. Res., 1923, **44**, 231.
[8] Jour. Am. Med. Assn., 1925, **84**, 723.
[9] Arch. Path. and Lab. Med., 1926, **2**, 59.
[10] Jour. Hygiene, 1909, **9**, 332 and 1910, **10**, 155.

patients of agglutinins for intestinal proteus-like bacilli. The so-called Weil-Felix[1] reaction takes advantage of this fact. They employ one of two strains isolated by them, called X 19 and have found that a positive agglutination in dilutions over 1 to 50 (macroscopic method) can be considered diagnostic. The reaction is usually positive by the sixth day. The agglutinin content usually continues to rise, so that titers of 1 to 1000 or more are common. The test should be repeated, if doubtful or if there is an associated positive Widal reaction. Bengston[2] and others have studied these strains and others of the proteus group and find that they agree in general characteristics (see large Table at end of Part I).

Other Diseases said to be due to Filtrable Viruses.—Several other diseases of less importance have been listed as belonging to those produced by a filtrable agent. (See Rivers' Compilation.)

Kruse[3] corroborated by Foster[4] reported that a filtrable virus is obtained from common colds, which would reproduce the disease. The work of Olitzky and Gates[5] on the etiology of influenza has not been corroborated.

The interesting discussion in regard to the nature of the transmissible lytic principle of Twort and of d'Herelle (see p. 68) brings up the question of the worth of an unlimited passage of an infectious material in establishing proof as to its being a living organism.

OTHER DISEASES OF UNDETERMINED ETIOLOGY.

Rocky Mountain Spotted Fever.—This is an acute infectious disease characterized by fever and a more or less hemorrhagic eruption. Some years ago Wilson and Chowning thought they found a protozoan in the blood similar to babesia in Texas fever. Their findings have not been corroborated. Their investigations proved, however, that rabbits are susceptible to the disease and that a tick of the genus Dermacentor probably carries the infection. Then Ricketts and Gomez made some very interesting studies on the disease. They found that guinea-pigs and monkeys are susceptible as well as rabbits, and they further found that in guinea-pigs and monkeys an attack of spotted fever produces a strong active inherited immunity characterized by a serum with high-protective but low curative power, and that the production of the serum in the horse with the use of serovaccination in man may give practical results. They found a moderate number of diplococcoid bodies in the blood of infected guinea-pigs and monkeys, and fewer in man. They found that the virus is transmitted by the infected female tick (wood-tick—Dermacentor venustus) to her young through the eggs. If the larvæ from these eggs are allowed to feed upon normal

[1] Wien. klin. Wchnschr., 1918, p. 11. Craig and Fairley: Lancet, 1918, **2**, 385.
[2] Jour. Inf. Dis., 1919, **24**, 428.
[3] München. med. Wchnschr., 1914, **61**, 1547.
[4] Jour. Am. Med. Assn., 1916, **66**, 1180.
[5] Jour. Exp. Med., 1920, vols. **21** and **22**.

guinea-pigs, these animals come down with the disease. Immense numbers of these apparent organisms are found in affected eggs and none were found at first in normal eggs. Afterward Ricketts found a few, but he thought these might be an avirulent species of the same organism. The salivary glands, alimentary sac and ovaries of infected female ticks are swarming with these bodies, while normal ticks seem to have none. Ricketts found that these bodies agglutinate with specific serum, 1 to 300 dilution. Wolbach[1] corroborated these findings and demonstrated a similar organism in characteristic lesions of experimental animals bitten by infected ticks. Frick[2] reported the finding of small bodies in centrifugalized blood cells, which he did not classify. Cumming[3] studied 2 cases occurring in California and reported a reaction in monkeys and an apparently specific swelling of the scrotum in a series of guinea-pigs from the blood of the original monkey.

Wolbach (1919) found groups of minute coccoid forms in endothelial cells of bloodvessel walls and in thrombi. All of these minute forms found in Rocky Mountain spotted fever and in the infected lice are classed with the forms found in typhus as Rickettsia. Those in Rocky Mountain fever are called Dermacentroxenus rickettsi. Wolbach and Scheinger[4] reported an increase of these bodies by cultural methods. Noguchi[5] showed that mixtures of the virus and immune rabbit serum confer complete immunity on guinea-pigs.

Chicken-pox.—Kling[6] claims to have been able to vaccinate against this contagious disease with the clear contents of a fresh vesicle in as early a stage as possible. He inserted the point of a sterile lancet into such a vesicle and then into the patient's skin, repeating six times. This work was corroborated by Rabinoff.[7] Hess and Unger[8] report immunity by a similar method.

Herpes Febrilis and Herpes Zoster.—An extensive study of these conditions was made by Goodpasteur and Teague,[9] and others. They corroborated the findings of Luger and of Lipschutz of intranuclear inclusions in experimental herpetic febrilis keratitis of rabbits and have demonstrated the inclusions in other parts of the animal, particularly the central nervous system. They believe that the inclusions represent the virus. They have demonstrated that the virus reaches the brain by way of the axis cylinders. They have produced experimental herpes zoster by inoculating the tarred skin of guinea-pigs and rabbits with the virus from man. The question of the relationship of herpes zoster to chickenpox and of herpes simplex or febralis to encephalitis has been the subject of much study. It is thought by the majority that each is a separate virus belonging to a group of similar viruses.[10]

[1] Jour. Med. Res., 1912, **28**, 1 (with bibliography); also 1916, **34**, 121 and **35**,147; 1919, **41**, 1; also Harvey Lecture, 1922.
[2] Public Health Rep., March 3, 1916, p. 516. [3] Jour. Inf. Dis., 1917, **21**, 509.
[4] Jour. Med. Res., 1923, **44**, 231. [5] Jour. Exp. Med., 1923, **37**, 605.
[6] Hygiene, 1913, **75**, 1032; Berl. klin. Wchnschr., 1915, **52**, 13.
[7] Arch. of Ped., 1915, **32**, 651. [8] Am. Jour. Dis. Chil., 1918, **16**, 34.
[9] See Filterable Viruses, already quoted, also Cowdry, Jour. Exp. Med., 1923, **38**, 1695.
[10] Jour. Med. Rec., 1923, **44**, 121, 139, 185; Am. Jour. Path., 1925, **1**, 29, 47.

Verruga peruana, a South American disease, has been shown by Strong and his co-workers[1] to be a disease distinct from oroya fever and to be produced by an unknown virus transmitted, according to Townsend,[2] by the bite of a gnat (Phlebotomus verrucanamun).

Noguchi[3] reported the cultivation of a pleomorphic microbe on semi-solid media, similar to the microbe he found connected with oroya fever.

[1] Jour. Am. Med. Assn., 1913, **61**, 1713.
[2] Ibid., 1913, **61**, 1717.
[3] Jour. Exp. Med., 1927, **45**, 175.

CHAPTER XXXIX.

PROTOZOA.

The classification and general characteristics of protozoa are given in Part I. The individual groups and pathogenic members will now be described.[1]

FLAGELLATA.[2]

The flagellates that are distinctly pathogenic to man belong to the genera Trypanosoma and Leishmania.[3] The other genera found in man may be mentioned because of the possibility of their becoming pathogenic.

Material and Methods for Study.—A number of flagellates are found in the large intestine of the lower animals. The feces are obtained by pressing lightly over the anus of the animal, or if the whole intestinal tract is to be examined, by sacrificing the animal and dissecting out the parts wanted. The material is placed in a clean watch-glass and thinned if necessary with physiological salt solution. Hanging drops may be made in physiological salt solution or in such a solution made a little thick by the addition of gelatin in order to retard the motion of the flagellates somewhat so they may be better studied.

Permanent preparations may be made according to directions given in Part I.

If one can obtain rats infected with Tr. lewisi, others with one or more pathogenic forms, the infecting organisms can be kept alive by frequent reinoculation of the heart's blood, subcutaneously or intraperitoneally into the fresh animal, or cultures may be carried on (see below). But generally one must rely on the permanent preparation. In the development in the second host one must also study the stained specimens in the majority of instances.

The fresh specimens of blood are obtained from the tail tip of the rat; they may be examined, after dilution with physiological salt solution, in the hanging drop. For permanent preparations films of the blood are spread, fixed and stained in the usual way; Giemsa's method of staining, or one of the modifications (see Stains), is very satisfactory.

For section work of the various organs the fixatives and methods given in Chapter III may be used. Special methods are given under each organism.

[1] Among the latest good protozoölogies see Knowles, An Introduction to Medical Protozoölogy, Calcutta, Thacker, Spink & Co., 1928; Craig, Manual Parasitic Protozoa of Man, Lippincott, 1926; Hegner and Taliaferro, Human Protozoölogy, Macmillan, 1924; Wenyon, Protozoölogy, London, 1926. Hegner gave a good summary of the transmission of human protozoa in Science, 1926, **64**, 28.

[2] See Part I for general description of Protozoa.

[3] Hartmann put the Trypanosomata, with other blood parasites, in an order, the Binucleata, and made the Spirocheta an appendix of this order. According to this arrangement the Hemosporidia are taken from the Sporozoa and placed in the Binucleata with the Trypanosomata. The malarial organisms supposedly lose through their parasitism many of the characteristics ascribed to this order. This order is not accepted by the majority of Protozoölogists.

Artificial Cultures of Blood Flagellates (see Chapter IV).—These, according to Novy and MacNeal,[1] modified by Nicolle[2] (the "N.N.N." medium), may be made on a culture medium consisting of a mixture of ordinary nutrient agar with variable amounts of fresh defibrinated rabbit or rat blood. The best all-around results are obtained with equal parts of blood and agar. The agar is melted and cooled at 50° C., then the blood is added and thoroughly mixed. The tubes are inclined until the medium stiffens, when they should be inoculated at once with blood or other infected material containing living trypanosomes. The surface of the medium should be very moist, so water of condensation may form. Generally evidence of growth may be observed in three or four days.

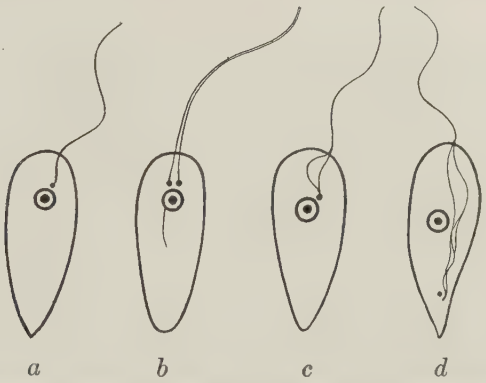


FIG. 182.—Schematic drawings of flagellates belonging to the trypanosomedæ, showing differential points: *a*, Leishmania; *b*, Herpetomonas; *c*, Crithidia; *d*, Trypanosoma.

LEISHMANIA, HERPETOMONAS, AND CRITHIDIA.

Certain flagellates found in the digestive tracts of mosquitoes, flies, and other insects are very similar to trypanosomes. Among them several species have been recognized, but they need to be more fully studied in order to determine their definite relationship to each other and to the genus trypanosoma. Leishmania is described as having a single flagellum directed forward and arising near a blepharoplast situated in the anterior part of the cell. Herpetomonas is almost indistinguishable from Leishmania. The flagellum containing two filaments which was thought distinctive is considered now to belong to dividing forms. A delicate filament extends from the blepharoplast toward the posterior end. Crithidia has a rudimentary undulating membrane. The distinctions between these three genera and the genus Trypanosoma which have been recognized are: (1) the former contain no undulating membrane or only a rudimentary one, and (2) their centrosome or blepharoplast usually lies at the side of, or anterior to, the nucleus instead of posterior to it, as in Trypanosoma (Fig. 182).

These distinctions, Novy claims, may disappear in the cultural forms of the three genera, when trypanosomes may show a rudimentary undulating membrane and an anterior blepharoplast. His caution in regard to confusing these insect flagellates with developmental stages of vertebrate blood parasites should be remembered.

[1] Contribution to Med. Res., dedicated to V. C. Vaughan, 1903, p. 549; also Jour. Inf. Dis., 1907, **4**, 223.

[2] Compt. rend. Acad. de sci., 1908, **146**, 842.

LEISHMANIA (LEISHMAN-DONOVAN BODIES AND ALLIES).

Certain fevers of severe malaria-like types known in different sections of the tropics by different names (dum-dum fever, cachexial malaria, kala-azar) have been shown to have a causal relationship by the finding of similar protozoön-like bodies in the lesions. These bodies were first minutely described by Leishman, in 1903, as being present in certain cells in the spleen of cases occurring in India, called by him dum-dum fever. He considered them as possibly trypanosomes, but did not name them. Later in the same year Donovan described similar bodies in cases of what he called malarial cachexia. The

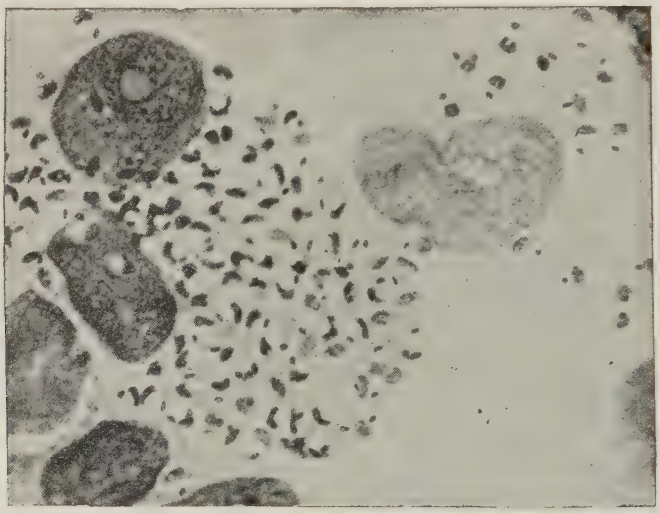


FIG. 183.—Leishmania tropica in a case of tropical ulcer. Smear preparation from the lesion stained with Wright's Romanowsky blood-staining fluid. The ring-like bodies with white central portions and containing a larger and a smaller dark mass are the microörganisms. The dark masses in the bodies are stained a magenta, while the peripheral portions of the bodies in typical instances are stained a pale robin's egg blue. The very large dark masses are nuclei of cells of the lesion. × 1500 approximately. (After Wright.)

bodies were first called the Leishman-Donovan bodies; then Laveran and Mesnil who examined Donovan's preparations and considered the organisms similar to those causing Texas fever in cattle, called them Piroplasma donovani. Ross, however, thought they constituted a distinct genus which he called Leishmania. This genus is now accepted, hence they are known as Leishmania donovani. Rogers and Patton place them with the genus Herpetomonas, but until we know more of the limits of variation of all these forms it seems best to make them a separate genus. They have since been found in different parts of India, in China, Tunis, Algiers, Arabia, Egypt, South Africa, Italy, Greece, Portugal and South America. Wright[1], in this country, reported in a

[1] Jour. Med. Res., 1903, 10, 472.

case of tropical ulcer, or Delhi boil, from an Armenian immigrant, bodies which, according to his excellent photographs (Fig. 183) and description, must be identical with, or very closely related to, Leishman's bodies. On account of the different pathological conditions in which they are found, however, they are classed as a different species, Leishmania tropica Wright. The form found in infantile splenomegaly is considered by some to be another species, Leishmania infantum Nicolle,[1] though Noguchi's work on agglutination points to this strain being related to the tropica. In South American tropics the disease called uta or espundia was shown by Lindenberg (1909) and others[2] to be caused by a Leishmania known as L. braziliensis. The diseases caused by these organisms are classed under the general name Leishmaniasis.

Morphology.—The bodies as seen in the cells of the host are circular to elliptical in shape, from 2μ to 4μ in diameter, and contain two nuclei, a large oval one at one part of the periphery and a small circular or rod-shaped one (blepharoplast) near or at the opposite part of the periphery. This smaller body stains more intensely than the larger one, while the cytoplasm of the parasite stains very dimly, sometimes showing only a faint peripheral rim. Near the rod-shaped nucleus may sometimes be seen a minute granule or rod which is the rudiment of the flagellum. Any nuclear and cytoplasmic staining methods will bring out these points in Zenker-fixed material. Smears stain well by Wright or the Nocht-Romanowsky methods. It was not until these organisms were cultivated outside of the body that their relationship to the flagellates was established (Fig. 184 and Plate IV, I, Fig. 1, B).

Site in Host.—The bodies have been found in large endothelioid cells in the spleen, liver, bone marrow, lymph nodes, kidney, lungs, testes, skin, muscles, intestinal ulcers and in the leukocytes in the peripheral blood. In this last situation they are only found in appreciable numbers inadvanced cases.

The large cells containing the parasites are supposed by Christophers to be the endothelial cells from the finest capillaries. Donovan states that he found small forms in the polynuclear leukocytes of the peripheral circulation when the temperature was above 103°. Marshall found them chiefly in the mononuclears. Rogers was able to demonstrate them more easily in the peripheral circulation by centrifugalizing the blood and examining the leukocytic layer. Nicolle and Conte and others obtained them in the mononuclears found in the fluid of blisters produced experimentally on the skin.

Cultivation.—Rogers has grown abundant pure cultures of the bodies in a slightly acid citrated blood medium at 20° to 22° C. Nicolle and later Novy have shown that L. infantum is pathogenic for dogs and other animals and that cultures may be obtained with comparative ease from the infected animals (Fig. 184). Nicolle has also cultivated L. tropicum.

In these cultures the rounded organism elongates, the flagellum

[1] Ann. de l'Inst. Past., 1909, **23**, 361 and 441.

[2] Strong and co-workers, Jour. Am. Med. Assn., 1913, **61**, 1713.

develops, and the Leishman-Donovan body becomes a flagellate, similar to Herpetomonas (Fig. 182). It divides by binary fission.

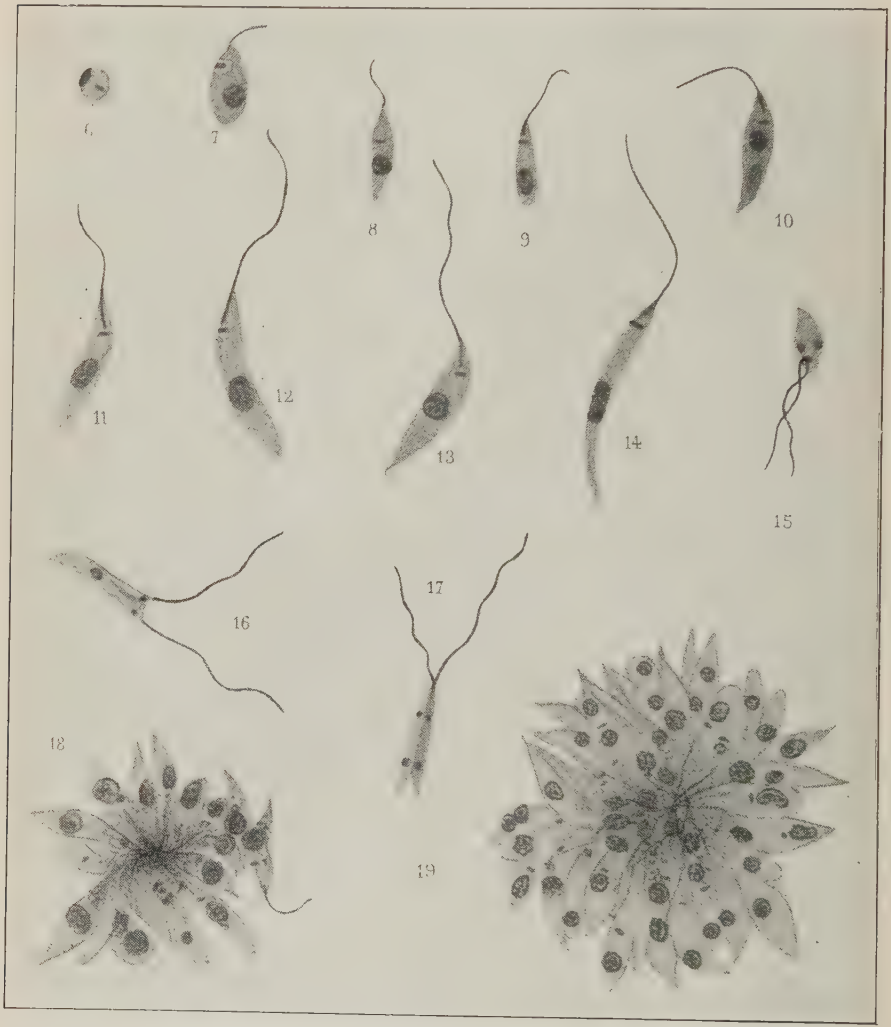


FIG. 184.—Cultural forms of *L. infantum*, showing the flagella type. (After Nicolle)

Del Aguila[1] reported the successful inoculation of the American Leishmania into the testicles of rodents.

Insect Carriers.—Rogers and Patton stated that the bed-bug may transmit the disease, and Patton[2] demonstrated the development of the

[1] Anal. d. l. Facul. Méd., June 1919, **2**, 42.

[2] Scientific Memoirs by Officers of Medical and Sanitary Departments of Government of India, N. S. No. 31.

organism up to the fully flagellated stage in the gut of this insect. But the bed-bug does not fit in with the epidemiology of the disease. The only insect that does fit is the sandfly. While sandflies had been suggested much earlier no positive evidence was obtained until 1921.[1] Then in 1925–1926 Adler and Theodor obtained more evidence,[2] which they think amounts to proof that the sandfly Phlebotimus papatasi (P. Sargenti) is the natural vector of oriental sore. Napier's[3] epidemologic studies of kala-azar pointed to the sandfly, P. argentipes, as the vector of this disease.

Effect on Human Host.—The pathological changes are those following the degenerations subsequent to the growth of the organisms in the large mononuclear cells. The *symptoms* in the cases of general infection are: (1) Very much enlarged spleen and less enlarged liver; (2) progressive anemia with peculiar, dark, earthy pallor of skin (kala-azar), progressive emaciation, and muscular atrophy; (3) long-continued, irregularly remittent, and intermittent fever (97° to 104°); (4) hemorrhages, such as epistaxis, bleeding from gums into subcutaneous tissue, producing purpuric eruption; (5) transitory edemas of various regions. There are often complications, such as congestion of lungs, dysentery, and cancrum oris. The blood shows practically no loss of red blood cells, but a diminution of hemoglobin; there is a decrease in the leukocytes, principally polynuclears, giving a relative increase of mononuclears.

Diagnosis. Negative points which help in the diagnosis are: Absence of malaria, no typhoid or Malta fever reaction, resistance to medication, quinine, as a rule, having no effect though in early cases and with large continued doses a few good results have been reported. Puncture of spleen or liver with the finding of Leishman-Donovan bodies makes the diagnosis certain. Sometimes the bodies may be found in the peripheral blood. Cultures on Novy and MacNeal medium may be obtained from each region.

The duration of the disease is from a few months to several years. The percentage of deaths in systemic infections is great; in some forms of the disease at the height of an epidemic it may reach 98 per cent. Natives seem to be more susceptible than strangers from non-tropical countries. The infection in children known as splenomegaly is similar to that in the adult.

The local disease known as Delhi or Aleppo boil or tropical ulcer is a comparatively non-dangerous circumscribed chronic ulcer in which the endothelial cells contain the organisms in large numbers (Fig. 183). Recovery is followed by marked immunity. Uta is a similar disease.

Prophylaxis.—Segregation and perfect cleanliness, especially in regard to sandflies or other biting bugs, are recommended as the best means of eradicating the disease.

Curative Treatment.—Continued favorable reports are coming of the use of tartar emetic introduced by Machado and Vianna in Brazil in both superficial[4] and internal[5] Leishmaniasis.

[1] The Sargents and co-workers.
[2] Am. Trop. Med. and Parasit., 1925, **19**, 365; **20**, 175.
[3] Ind. Med. Res. Mem., 1926, **4**, 219.
[4] Da Malta Amaz. Med., 1918, **1**, 13.
[5] Pastore, Pediatria, 1919, **27**, 96.

CHAPTER XL.

TRYPANOSOMA.

Pathogenic Forms.—Very many species of trypanosoma have been described, and the number reported as distinctly pathogenic is increasing. Two of the latter are known to be pathogenic for man; a closely related form that was described by Chagas in 1909 is made a new genus. The table on page 629 gives a list of the better-known forms pathogenic for mammals with their chief differential characteristics. They are divided by Laveran into three groups according to the different characters of the flagellum. Their general characteristics and broader classifications are given in Part I. (See also Plate IV, 1, Fig. 2.)

Historical Note.—The first species of trypanosome studied with any degree of fulness is the comparatively non-virulent T. lewisi. It was probably first seen in the blood of the rat in 1845, but was not well described until 1879, when Lewis studied it more fully. Since then it has been studied by many observers. It is found in the blood of from 2 to 3 per cent. of wild rats throughout the world.

The first of the more pathogenic trypanosomes was discovered by Evans in the blood of East Indian horses suffering from surra, but it was not well studied until 1893, when Lingard's important work on *surra* led, in a way, to all the subsequent work on diseases caused by trypanosomes. The next year a trypanosome was discovered by Bruce[1] in the blood of horses and cattle suffering from nagana in Zululand and other parts of Africa. Bruce further demonstrated the important fact that the disease was transmitted by the bites of flies, the tsetse flies (*Glossina*). Announcements of other pathogenic trypanosomes in different parts of the tropics quickly followed. In 1896 Rouget found that *dourine*, a disease of equines in Algiers and South Africa, was caused by a trypanosome (T. equiperdum). Then the South African disease of horses, called *mal de Caderas*, was shown by Voges to be due to a similar flagellate, while in 1902, Theiler found a variety of trypanosome in the blood of cattle in the Transvaal suffering from the disease called *galziekte*, or gall sickness. The number of trypanosomes found in the tropics is constantly increasing—both pathogenic and non-pathogenic forms.

Man was thought to be comparatively immune to trypanosomes until the important discovery was made that trypanosomes are the specific cause of a definite disease known as *sleeping sickness*, which occurs chiefly in the African negro. In 1898 Nepvieu reported having found trypanosomes in the blood of 6 out of more than 200 cases of human beings examined for malarial organisms and in the seventh case which was apparently in good health. In 1901 and 1902 Dutton and Todd made an extensive study of natives in Africa and found trypanosomes in the blood of 6 of them. The tenth and eleventh cases were published by Manson in 1902, Bröden published 2 more cases and Baker 3. In 1903 Castellani found trypanosomes in the centrifugalized cerebrospinal fluid of 20 out of 34 cases of this disease. His work has been fully corroborated. The trypanosomes found in these cases resemble each other; they are therefore included under the same name, Trypanosoma gambiense Dutton. A similar form found by Stephens and Fantham[2] in 1911 in cases of sleeping sickness in

[1] British Med. Jour., 1915, Nos. 2843–2846

[2] Proc. Roy. Soc., 1910, **88**, 28.

DIFFERENTIAL CHARACTERISTICS OF MORE IMPORTANT TRYPANOSOMES PATHOGENIC FOR MAMMALS (MODIFIED FROM YORKE AND BLACKLOCK).

Groups	Sub-groups.	Species.	Date discovered.	Where first found.	Size in microns. Length. Max.	Length. Min.	Breadth. Max.	Breadth. Min.	Other characteristics.	Pathogenesis. Vertebrate host.	Name of disease.[1]	Laboratory animals.	Invertebrate host. Name.	Place of development.
I. Always free flagellum.	1	T. lewisi Kent	1881	Throughout world	38	10	3.0	1.5	Very motile, nucleus at anterior and middle third, blepharoplast rod-shape.	Rats	Trypanosomiasis	Rat slight	Rat fleas (Ceratophyllus) and lice (Hæmatopinus)	Intestines.
		T. evansi Steele	1885	India	34	18	2.0	1.5	.....	Equidæ, cattle, camels	Surra	Acute	Stomoxyx (fly)	Unknown.
	2	T. brucei Plimmer and Bradford	1899	Zululand	34	18	2.5	1.5	Post. end usually bluntly rounded	Equidæ, cattle	Nagana	Acute	Glossina	Gut and salivary glands.
		T. hippicum Darling	1910	Central America	28	18	3.0	1.5	.....	Equidæ	Trypanosomiasis	Acute	.....	Unknown.
	3	T. equiperdum Doflein	1901	North Africa	35	16	3.0	1.5	.....	Equidæ	Dourine	Acute		Transmitted by coitus.
	4	T. vivax Ziemann	1905	Cameroon	31	16	3.0	2.0	Very motile, club shaped	Equidæ, cattle	Trypanosomiasis		Glossina	Proboscis.
	5	T. theileri Laveran	1902	German East Africa	70	25	5.0	2.0	Very large	Equidæ, cattle	Trypanosomiasis		Tabanid fly	
	6	T. (Schizotrypanum) cruzi Chagas	1909	Brazil	Average		20.0		Dividing forms not seen in blood, blepharoplast large	Man, monkey	Trypanosomiasis	Slight	Triatoma	Gut and salivary glands.
II. Never free.	7	T. congolense Borden	1904	Congo	19	8	Average	1.5	.....	Equidæ, ruminants	Trypanosomiasis	Acute	Glossina	Proboscis.
	8	T. simiæ Bruce	1912	Nyasaland	23	12	..		.....	Monkeys	Trypanosomiasis	Acute for monkeys and pigs	Glossina	Proboscis
III. Free or not.	9	T. gambiense Dutton	1902	Gambia	39	13	..	..	Moderately active	Man	Sleeping sickness	Acute	Glossina	Gut and salivary glands.
	10	T. rhodesiense Stephens and Fantham	1910	Rhodesia	39	13	..	.	Virulent for rats	Man	Sleeping sickness	Acute	Glossina	

[1] The general name is, of course, trypanosomiasis.

Rhodesia has been given another specific name, T. rhodesiense, chiefly because of the characteristic action in experimental animals.

Chagas,[1] in 1909, states that a trypanosome (T. cruzi) which he had discovered in a small monkey (Callethrix hapalepenecellata) is the cause of human infection (Chagas' disease) in Rio de Janeiro. Because of its ability to grow in the tissues of infected animals producing Leishmania-like forms, it is classified by some as a new genus—schizotrypanum. It is carried chiefly by the bug Triatoma megista. The flagellate is small with a large blepharoplast (kinetonucleus). It grows on blood agar readily and infects laboratory animals easily. Chagas reports developmental forms in the monkey's lung and in the gut of the fly. Chagas has also reported that this trypanosome causes thyroiditis in children. Tejera[2] reports similar infections in Venezuela, transmitted by a different insect (Rhodnius prolixus). Armadillos, cats, dogs and monkeys are reservoir hosts of the infection for man.

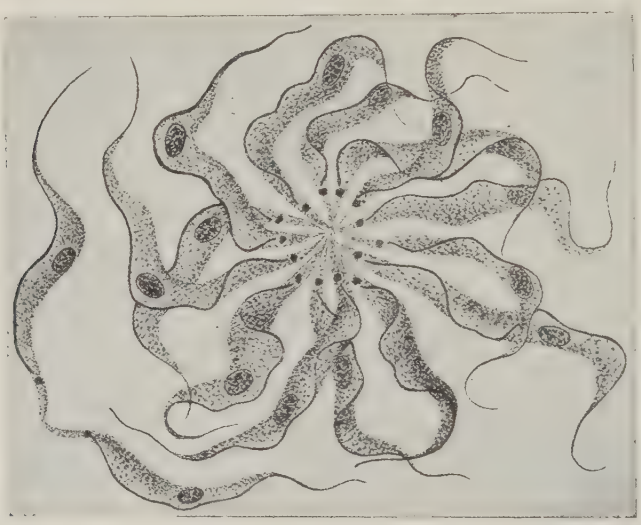


FIG. 185.—Agglutination of Trypanosoma lewisi. (Laveran and Mesnil.)

Comparative Characteristics of the Different Species.—The form changes of the same species in the same host are so varied that few have been found absolutely characteristic of a single species, and, as physiological properties alone are not considered final in species classification, we cannot be sure that all of the organisms in this group described as separate species are so until more of the complete life histories are known. Until then each new form found with distinct physiological properties, though apparently morphologically similar to others, may expediently be considered a new species.

As an example of the difficulty of deciding whether or not a trypanosome found is a new species, the history of T. rhodesiense may be taken. All of the points brought forward by Stephens and Fantham, the discoverers, as evidence of a new species have been combated by others; and its relation to T. gambiense on one side and T. brucei on the other is an open question. At present the points are in favor of its being the same as T. brucei.

[1] Mem. Inst. Oswald's, orig., 1909, **1**, 159; also Report of Lecture given in 1921 in New York City.

[2] Gac. Méd. d. Caracas, 1919, **10**, 103.

Morphology.—**Size.**—The variations recorded in the *dimensions* of the species we are considering may be seen by glancing at the foregoing table. The trypanosomes pathogenic for man (*T. gambiense* and *T. rhodesiense*) have the smallest average size of the group. With the exception of *T. theileri* and *T. transvaaliense* (not given in table), which are much larger than any other of these forms, the variations in size of the different species are not so marked as they are in the same species under different conditions.

Shape.—In shape, though all follow the type, each species varies greatly according to conditions of growth and multiplication. At times they may be slender and worm-like, at others they may be so short and thick as to be almost round. *T. lewisi* has the posterior (aflagellar) end often thinner and more pointed than the other species. *T. evansi* is generally a little longer and thinner than *T. lewisi*, while *T. brucei* has a more rounded aflagellar end than either, and is generally broader, more club-shaped (Plate IV, Figs. 1, 2).

The Cytoplasm.—The cytoplasm differs slightly in the different forms. *T. lewisi* is relatively free from chromatoid granules, while *T. brucei* has usually many. Myoneme fibrils have been demonstrated in some species and probably all contain them. An oval vacuole has been seen in some species.

The Nuclear Apparatus.—The nuclear apparatus is essentially similar in all forms. The two nuclei (tropho- and kinetonucleus) vary somewhat in position and size in the different species and at different stages in the same species. In *T. theileri* and in young forms of *T. lewisi*, both nuclei lie close together near the center of the organism. In *T. lewisi* the trophonucleus is situated more anteriorly than in the other species. In *T. rhodesiense* the nucleus is often posterior to the center in experimental animals. Bruce thinks that *T. rhodesiense* is a slightly modified *T. brucei*.

Many variations from the type forms are seen. Some are no doubt degeneration and involution forms. *Three* forms, however, which are more or less constantly seen in all the species have been interpreted as definite phases in the life cycle. These forms were first described by Schaudinn in T. noctuæ, and were interpreted by him and since then by others as male, female, and indifferent form. The male cells are smaller, more hyaline, and more free from granules than the female. The nucleus of each sexual cell rids itself of male and female chromatin, respectively. The indifferent cell, on the contrary, has a complete nucleus. Opinions differ as to Schaudinn's interpretation being the correct one. More research is needed before we can arrive at a definite conclusion.

Motility.—The first thing noticed on examining a fresh hanging drop of blood at a magnification of 100 to 300 diameters is active movements of the red blood corpuscles in certain areas, and, on carefully focussing over one of these areas, the rapidly wriggling worm-like organism may be seen. As the movements become slower, the flagellum may be seen swaying from side to side and the wave-like movements of the undulating membrane are quite discernible. Movement is twofold: (1) progression with an auger-like motion effected by the

undulating membrane assisted by the flagellum; (2) contractions of the body assisted no doubt by myoneme-like structures. Relatively, *T. lewisi* is most active and *T. brucei* least. Motility soon ceases outside of the body, continuing longer if the organism has been kept in the ice-box than at higher temperatures. Aflagellar forms, sometimes ameboid, have been frequently described in the blood of mammals.

Reproduction.—The usual method of multiplication is binary longitudinal fission. In several species a rosette-like segmentation has also been observed. Longitudinal fission begins usually with division of the kinetonucleus, then of the trophonucleus and cytoplasm; but this order of division seems to be quite variable. The flagellum often appears to be dividing first, and probably division always starts with the centrosome-like basal granule of the flagellum. In many cases a new flagellum seems to be formed instead of division of the old one. The details of division have not been frequently studied, but it is probable that both nuclei divide by a primitive mitosis. During division the kinetonucleus generally moves near the trophonucleus. Generally the fission is equal, but occasionally the daughter trypanosomes may be quite unequal in size. This is notably the case in division of *T. lewisi* where the cytoplasm may divide so unequally that the process may be compared to budding. The resulting small parasites have at first no undulating membrane, hence they resemble somewhat Leptomonas. These young forms may divide several times in succession, producing smaller and smaller fusiform parasites. As a result some forms are so small that they can only be seen when agglomerated or in motion (Schaudinn). The question as to whether trypanosomes undergo phases of development in their invertebrate hosts has been widely studied. Minchin and Thomson[1] gave a good report of the life history of *T. lewisi* in the rat flea, the full working out of which was the result of the investigations of a number of workers.

Insect Carriers.—The trypanosomiasis of vertebrates is transmitted by blood-sucking insects (dourine is possibly an exception). Bruce (1894) first showed that *T. brucei* was conveyed by the fly *Glossina morsitans*. Since then other varieties of flies also have been shown to spread the disease (table, p. 629). Among them *Glossina palpalis* (Fig. 186) is supposed to be the chief agent in transmitting *T. gambiense*. These flies bite by day and in full moonlight. The infective period of the insects after they have bitten a sick animal is variable. Bruce found living trypansomes in the proboscides of the flies up to forty-eight hours. Up to one hundred and eighteen hours he found them in the flies' stomachs, after one hundred and forty hours he found the stomachs empty, and what appeared to be dead parasites in the excreta. Klein, Robertson,[2] and others since found that after a certain number of days (usually from fifteen to thirty) a small percentage of the biting flies which were fed sufficiently on an infected animal become infective again. This is due to the fact that the trypanosomes have developed

[1] Quart. Jour. Med. Sci., 1915, **60**, 463.
[2] Phil. Trans. Roy. Soc. London, 1913, (B), **203**, 161.

in the gut and have passed to the salivary glands of the fly. They remain in the glands probably during the life of the fly, and continue to be infective (Bruce, Hammerton, Bateman, Mackie,[1] and others).

Dourine in horses is usually communicated during the act of coitus. Surra may be transmitted to dogs by eating infected animals. The rat trypanosome is conveyed by contaminative infection through the dejecta of the fleas or lice when biting a new host. (See also under *historical note.*)

Cultivation.—Novy and MacNeal[2] were the first (1903) to cultivate trypanosomes in the test-tube. They have grown *T. lewisi* through many culture generations extending over several years. At the last tests made the parasites were as virulent as at the beginning. The

Fig. 186.—Glossina palpalis, carrier of the human trypanosomiasis caused by T. gambiense. × 4. (Kolle and Wassermann.)

culture medium used in their work was the condensation fluid from slant tubes of ordinary nutrient agar containing variable amounts of fresh defibrinated or laked rabbit or rat blood. The best results were obtained with a mixture of equal parts of blood and agar. At room temperature the growth is slower but surer than in the thermostat. A culture at room temperature retains its vitality for months; thus in one case the trypanosomes were alive after three hundred and six days. Novy and MacNeal also cultivated *in vitro T. brucei, T. evansi,* and various bird trypanosomes. The latter they found especially easy to cultivate, while the former are much more exacting in their require-

[1] Proc. Roy. Soc., 1911, **81**, 405.
[2] Jour. Inf. Dis., 1904, **1**; 1905, **2**, 256.

ments than is the *T. lewisi.* They require two parts of blood to one of agar, and growth is best at 28° C. The primary cultures are not transferred until the end of three weeks. These primary cultures are not always virulent for animals, but subcultures regain their virulence. Mice and rats die in six to fourteen days after inoculation. Guinea-pigs have a relapse in two to ten weeks.

The great majority of trypanosomes experimented with since the initial investigations have been found by various workers to be cultivatable with more or less ease. *Trypanosoma gambiense,* however, seems to be more difficult to cultivate (Thompson and Sinton[1]).

Effect on Vertebrate Host (Pathogenesis).—Lower Animals—Many of the lower vertebrates have become, through mutual toleration, natural hosts of the trypanosome. It is probable that each pathogenic trypanosome has an indigenous wild animal as natural host and that in this way the supply to strange mammals coming into the vicinity is kept up. These strange animals, being unaccustomed to the native trypanosomes, often succumb to the infection.

Symptoms.—In general the descriptions given of the symptomatology of trypanosomiasis in various animals show a great similarity, though there is much variation in individual cases. The average clinical picture, according to Musgrave and Clegg,[2] is as follows: After an incubation period which varies in the same class of animals and in those of different species, as well as with the conditions of infection, and during which the animal remains perfectly well, the first symptom to be noticed is a rise of temperature. For some days a remittent or intermittent fever may be the only evidence of illness. Later on the animal becomes somewhat stupid; watery, catarrhal discharges from the nose and eyes appear; the hair becomes roughened and falls out in places and the peripheral lymph nodes are enlarged. There may be various ecchymoses and skin eruptions. Finally, the catarrhal discharges become more profuse and the secretions more tenacious and even purulent; marked emaciation develops; edema of the genitals and dependent parts appears; a staggering gait, particularly of the hind parts, comes on, in some forms passing on to paralysis. This is followed by death. Parasites are found in the blood more or less regularly after the appearance of the fever. They are often more numerous in the enlarged lymph nodes and in the bloody edematous areas than in the general circulation. They begin to die just before the death of the host.

The *autopsy* shows general anemia, an enlarged spleen with hypertrophied follicles, more or less gelatinous material in the adipose tissue, the liver slightly enlarged, a small amount of serous exudate in serous cavities, edematous condition, and small hemorrhages in various tissues. There is a relative increase of the mononuclears in the blood.

The *duration* varies from a few days to many months. The *prognosis* seems to be influenced to a certain extent by the species of host. It is probably always fatal in horses. Some cattle recover. The chief cause of death is possibly a toxic substance, though no definite toxin has been isolated. Mechanical disturbances (emboli, etc.) also probably play a part in producing death.

Man.—*Sleeping sickness,* or *human trypanosomiasis,* is an endemic disease in certain regions of equatorial Africa. Neither age nor sex are predisposing factors, but occupation and social position seem to have a marked influence, the great majority of cases occurring among

[1] Ann. Trop. Med., 1912, **6**, 351.

[2] Trypanosoma and Trypanosomiasis, etc., Manila, Bureau of Public Printing, 1903.

very poor field workers. As these workers are all negroes, the question of the relative influence of race cannot be determined. The white race, however, is not immune, as has been frequently shown.

T. gambiense, the trypanosome first shown to be pathogenic for human beings, is irregularly pathogenic for some monkeys (Macacus rhesus and others), for dogs, cats and rats. It is less pathogenic for mice, guinea-pigs, rabbits, horses, baboons, cattle and swine. T. rhodesiense is more pathogenic for test animals.

Flies (G. palpalis) have been found to be infected with T. gambiense in areas which have had no human population for several years, a longer time than the life of a fly. And since trypanosomes are not known to be hereditarily transmitted, it is concluded that certain mammals are harboring the trypanosomes, thus acting as reservoirs.

Symptoms.—The course of the disease is very insidious, as the trypanosomes may exist in the blood for a long time before entering and growing in the cerebrospinal fluid and causing the characteristic symptoms. Therefore the symptoms may be divided into two stages. In the first stage there is only an irregular fever with enlargement of the peripheral lymph nodes. In the second stage the fever becomes hectic, the pulse is constantly increased; there are neuralgic pains, partial edemas and erythemas, trembling of the muscles, gradually increasing weakness, emaciation, and lethargy. The somnolence increases until a comatose condition is developed and death occurs. In the second stage trypanosomes are always found in the cerebrospinal fluid. Throughout the disease they are usually found in small numbers in the blood.

Duration.—The first stage may last for several years; the second, from four to eight months. The percentage of deaths in cases reaching the second stage is 100. Whether some in the first stage recover is not yet certain.

Pathological Changes.—Congestion of the meninges; increased quantity of cerebrospinal fluid; hypertrophy of spleen, liver, and lymphatic ganglia; diminished hemoglobin and number of red cells; number of leukocytes about normal, but a relative increase of eosinophiles, mast cells, and lymphocytes. Enlargement of the superficial lymph nodes has been noted as an early symptom and has thus been made use of in diagnosis. Dutton and Todd found that 91 per cent. of natives in the Congo Free State, who had posterior cervical glands enlarged, showed trypanosomes in the punctured gland juice.

Diagnosis of Trypanosomiasis in General.—This should be made as early as possible in order to prevent the spread of the disease. An early positive diagnosis can only be made by the determination of the peripheral infection. This is done in two ways: first, by microscopic examination of freshly drawn blood, cerebrospinal fluid, or tissue from enlarged peripheral lymph nodes; second, by animal inoculation of the blood or other tissue. In the microscopic examination it may be necessary to examine the blood of the suspected animal for several days in succession. The parasites are rarely absent in the early stages in domestic animals for more than a few days at a time, while in man the time may be much longer.

Methods of Examination.—Blood.—If the direct examination of a drop of the blood is negative, 10 c.c. should be withdrawn from the vein, and after adding a tenth of its volume of 10 per cent. citrate of sodium it should be centrifugalized for ten minutes, and the sediment examined in hanging drop and in smear. The great majority of the parasites will be found collected with the white cells in the thin white layer between red blood cells and serum, which may easily be removed with a fine pipette. The parasites are readily detected with the low power of the microscope

(× 100) by areas of irregularly moving cells. If only a small amount of blood can be obtained, the tiny tubes recommended by Wright in his opsonin work may be used.

Cerebrospinal Fluid.—Ten c.c. of the fluid withdrawn by lumbar puncture should be centrifugalized for fifteen minutes, and the deposit should be examined under 150 to 200 diameter magnification.

Cultures sometimes give positive results, especially from infections in test animals.

The Inoculation Test.—If the trypanosomes cannot be found by the above methods, animal inoculation should always be made. Monkeys, if possible, should be used, or if monkeys cannot be obtained, dogs, rats or guinea-pigs may be used. A few drops to 1 c.c. of the blood or other tissue from the suspected animal should be inoculated intraperitoneally or subcutaneously.

Smears may be stained by any modification of the Romanowsky method. Giemsa's method gives good results (Plate IV, Figs. 1, 2).

Prophylaxis.—The disease is readily controlled by preventive measures. There should be strict quarantine regulations governing the importation of animals. When the disease has once appeared, the following general measures should be taken: (1) Suspected animals should be isolated. (2) All infected animals should be destroyed. (3) As far as possible, all biting insects should be destroyed, and the land in the neighborhood of human habitation should be suitably cultivated. (4) The bodies of infected animals should be protected from biting insects for at least twenty-four hours after death. (5) Susceptible animals should, if possible, be made immune. (6) All means possible should be used to exterminate the reservoirs. In sparsely settled districts the natives may be removed from the fly area.

Treatment.—The whole question of treatment is still in the experimental stage. The chronic course of the disease with relapses often after long intervals makes it impossible, especially in cases of human trypanosomiasis, to come quickly to a conclusion in regard to the efficiency of any drug. Many drugs have been found to possess trypanocidal properties to a certain extent.

Atoxyl (p-amino-phenyl-arsenic acid), introduced by Thomas (1905) and used first by Thomas and Breinl in treatment of experimental trypanosomiasis, proved to have a beneficial effect in the different forms of this disease.

The good reports received from the use of other arsenic compounds introduced by Ehrlich,[1] namely salvarsan and neosalvarsan, have continued. Bayer 205 is giving very promising results. Chagas' disease, however, seems not to be influenced by any of these drugs.

Serum Therapy.—Various normal sera from different animals have been tried with practically no success. A few have prolonged life. Thus Laveran and Mesnil[2] state that human serum injected in sufficient quantities shows manifest action on the disease, and that sometimes cure results in mice and rats. Further, by alternating human serum

[1] München. med. Wchnschr., 1909, 5, 217.

[2] Trypanosomes and Trypanosomiases, Trans. by Nabarro, London, 1907.

with arsenic they obtained still better results. Kanthack, Durham, and Blandford showed that animals recovering from trypanosoma infection were immune to further infection. Rabinowitsch and Kempner have made a very careful study of immune serum produced by *T. lewisi*. They have shown that an animal may be hyperimmunized and that then its serum, in comparatively large doses, inoculated into mice at the same time as the trypanosomes, or twenty-four hours before or after, allows no development of the organisms. Laveran and Mesnil state that the serum causes the rapid destruction of the organisms by the leukocytes, though MacNeal,[1] on the other hand, states that the trypanosomes are destroyed by a cytolytic action of the serum. This immune serum also has a similar action on the trypanosoma of dourine. The serum of animals hyperimmunized against other varieties of trypanosoma is not as active as that obtained by the inoculation of *T. lewisi*. Koch suggested that immunity might be produced by the inoculation of attenuated parasites, and Novy and MacNeal succeeded in attenuating cultures of *T. brucei*, and obtained some success in protecting experimental animals against virulent cultures.

Complement Fixation.—The earlier tests did not promise practical results. Several investigators, however, have given more favorable reports. In this country Mohler claims that the test is of great worth in diagnosing dourine, many cases of which have occurred in some of our western States (notably Iowa and Montana). Mohler, Eichhorn, Buck and Traum[2] state that they use a fresh antigen prepared from the spleens of rats dead after infection with surra (used because it is easier to transmit than is dourine). The antigen is simply a filtered emulsion in salt solution of the infected spleen. The emulsion from each spleen is made up to 40 c.c. by addition of salt solution. A fresh antigen must be prepared and titered (see Part I) each day. The results have been controlled by autopsies of animals giving a positive reaction.

[1] Jour. Inf. Dis., 1904, 1, 537.
[2] Jour. Agric. Res., 1913, 1, 99.

CHAPTER XLI.

OTHER FLAGELLATES FOUND IN MAN.[1]

Three genera among the flagellates—Chilomastix, Trichomonas and Giardia—are found frequently in the intestinal contents of man. Two other genera—Embadomonas and Enteromonas—have been reported as occasionally present.[2] A few other forms, called Coprozoic protozoa (free-feeding dung-feeding forms), may gain access to human feces from time to time. The commonest of these is Bodo caudatus. Dobell and others think that none of the intestinal flagellates are pathogenic, but that they merely increase in certain pathogenic conditions. Many, however, think that members of the second and third genera may be pathogenic. A small form called Waskia intestinalis, but classed as an Embadomonas by Castellani and Chalmers,[3] was described by Wenyon and O'Connor in 1917. Hogue[4] isolated it from a patient with chronic diarrhea, and has the form in cultivation on three different liquid media, of which the Locke egg seems the best. This was the first time that cysts of intestinal flagellates had been obtained in cultures.

The following is a brief description of each of these three forms.

Chilomastix.—This genus was not clearly differentiated from other intestinal flagellates until the work of Wenyon (1909–1910). It had no doubt been described under other forms, such as cercomonas and trichomonas. Wenyon's species was called C. mesnili. It seems to be a widely distributed inhabitant of the large intestines of man. It is a pear-shaped organism from 7 to 24μ long, and about one-third of the length in transverse diameter. It has three anterior flagella, and a fourth one lying inside the oral cavity. Kofoid and Swezy[5] have made a minute study of this species.

Trichomonas.—Donné, in 1837, described a form which he found in the human vagina, and which he therefore called *Trichomonas vaginalis*. It has been found by other observers to be a frequent habitant of the vagina at all ages. It is also found occasionally in the acid urine of both sexes. The mode of infection of the female is unknown. The body of the parasite at rest is pear-shaped, but during action its amœboid movements cause it to assume various shapes. The size varies from 12μ to 25μ long and 8μ to 15μ wide. The protoplasm is finely granular, excepting for two rows of larger granules which begin on either side of the nucleus and converge posteriorly. From the anterior part project four flagella, which seem to begin at a basal thickening near to, or connected with, the more or less oval, indistinctly vesicular nucleus. From the origin of the flagella an undulating membrane extends backward. The

[1] Hegner: Intestinal Protozoa and Host-parasite Relations in Newer Knowledge, etc. Ed. by Jordan and Falk, Univ. of Chicago, Press, 1928.

[2] Dobell: The Intestinal Protozoa of Man, London, 1921.

[3] Manual of Tropical Medicine, 1919.

[4] Jour. Am. Med. Assn., 1921, **77**, 112; Am. Jour. Trop. Med., 1928, **8**, 325

[5] Univ. Calif. Pub. Zoöl., 1920, **20**, 117.

body also seems to possess a certain linear structure connected with the membrane. Contractile vacuoles have not been seen.

Trichomonas Hominis Davaine.—This form, found frequently in the human alimentary canal, is very similar to the Trichomonas vaginalis, but it is smaller and more pear-shaped. It has been found often in acute diarrheas, and the causal relation between it and the pathological process has been accepted. Since the Great War many cases of trichomoniasis have been reported.

FIG. 187.—Trichomonas vaginalis. (Blochmann.)

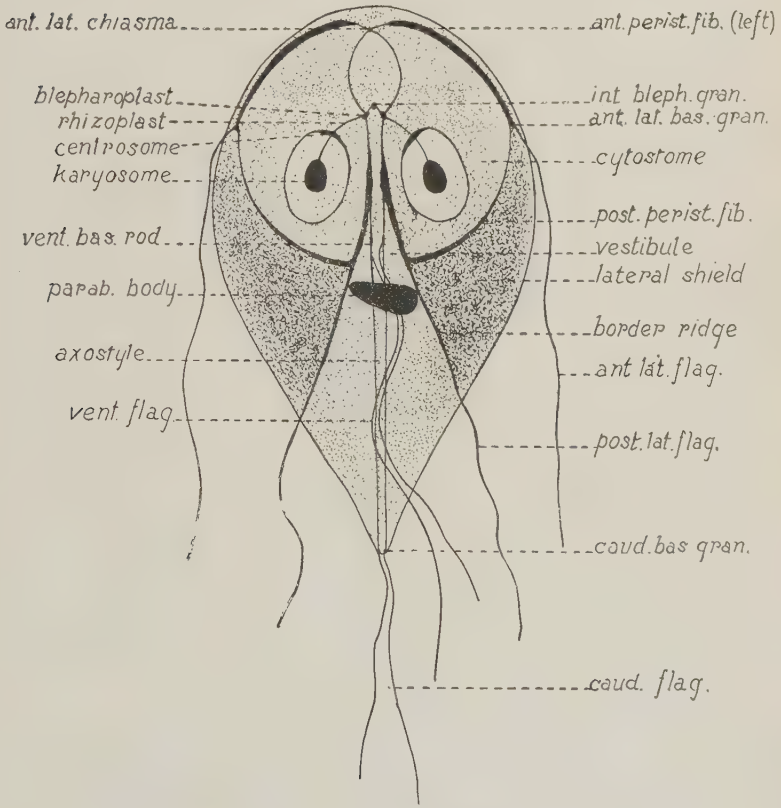


FIG. 188.—Giardia lamblia. Adult flagellate. (After Simon.)

A similar form has been seen a few times in lung gangrene, aspiration pneumonia, and bronchiectasis. A similar form has been found in the mouth (*Trichomonas buccalis*) by many investigators.

Trichomonads with three flagella (tritrichomonas) and five flagella

(pentatrichomonas) have been described as occurring more or less frequently in the alimentary canal of man. The latter seems to have the faculty of ingesting red blood cells. It has been found quite often in diarrhea.

Giardia lamblia, Stiles, 1915 (*Lamblia, intestinalis*, Lambl, 1859).—Flagellates belonging to this group are parasitic in the small intestines of mice, rats, rabbits, dogs, cats and sheep, and human beings. Both the generic and specific names in the group are very much confused. Stiles[1] has given a good history of the subject. The organism is beet-shaped, bilaterally symmetrical, 10μ to 21μ long and 5μ to 11μ wide, possessing flagella 9μ to 14μ long. Anteriorly, this species has a characteristic concavity, the rim of which seems to be contractile, forming a sucking apparatus. The eight flagella of the organism are arranged in pairs: one anteriorly, two laterally, and one posteriorly. The nucleus

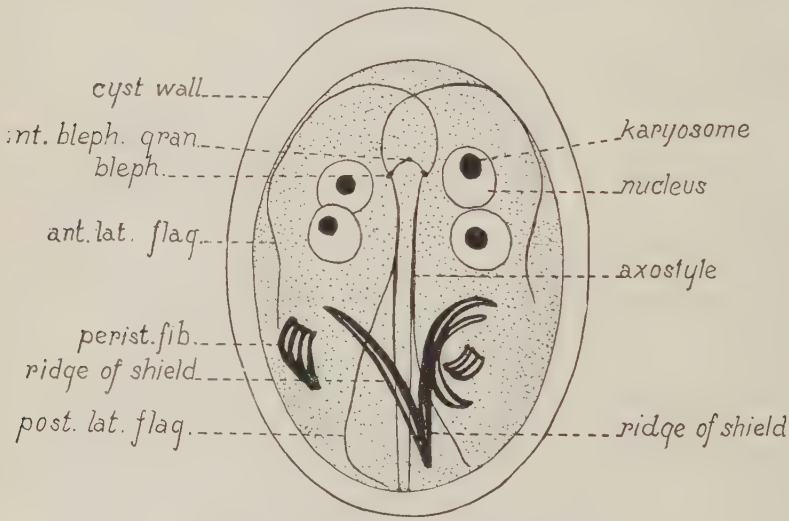


Fig. 189.—Giardia lamblia. Cyst. (After Simon.)

is situated anteriorly and has a central constriction. The protoplasm of the body is thick and hyaline. Contractile vacuoles have not been seen. Schaudinn (1906) observed encystment, copulation, and complicated nuclear changes in this organism. Simon[2] has reported a detailed study of this form.

Infection follows the ingestion of the cysts with unclean food. The parasites fasten themselves to the free surfaces of the epithelial cells by their sucking apparatus, but seem to exert little harmful influence on their hosts. They have been found most frequently in poor children who play often in dirt containing the cysts. A number of adult cases of apparent Giardiosis have been reported by Labbé and others.[3] Repeated small doses of calomel will usually cause their disappearance from the feces.

[1] Hygienic Laboratory, Bull. No. 133, 1923, p. 175.
[2] Am. Jour. Hyg., 1921, 1, 440.
[3] Labbé: Presse Méd., 1919, **27**, 161.

CHAPTER XLII.

AMŒBÆ.

Several authors early reported the finding of amœbæ in man, especially in so-called tropical, ulcerative, or amœbic dysentery, but as the first descriptions were incomplete and the laws of nomenclature were not strictly followed there resulted as usual many synonyms for the same species and a number of species bearing the same name.

At present four genera are described as containing species that are parasitic in man. These are Endamœba, Endolimax, Iodamœba, and Dientamœba, three species in the genus Endamœba and one each in the other three genera. Only one species among them is described as pathogenic—Endamœba histolytica.

Historical Note.—Stiles[1] has given a detailed history of the naming of the the entozoic amœbæ. This article illustrates very forcibly the absurdity of bringing forth new names for organisms only half studied and of claiming that such organisms belong to new genera.

The first entozoic amœba described as occurring in man appears to be the form called Endamœba gingivalis which Gros (1849) found in the tartar of the teeth. The first description of an intestinal amœba was probably by Lewis (1870) followed by Cunningham (1871). Supposedly the same forms were more fully described by Lösch, in 1875, under the name Amœba coli. Lösch found his organisms in stools of a patient suffering from chronic dysentery, and he succeeded by rectal injections in producing superficial ulceration in the large intestines of dogs. He therefore claimed that this organism is the cause of dysentery. His work was corroborated by many observers. In the meantime amœbæ were found in diseases other than dysentery, and Grassi, in 1879, reported them in healthy intestines. The work of Kartulis[2] (1886), however, helped largely to establish the fact that amœbæ play an important part in the etiology of dysentery in Egypt. He was the first to find the organism in abscess of the liver in tropical dysentery. In our own country among the most important workers in this field were Councilman and Lafleur[3] (1891). They concluded that amœbic dysentery should be regarded etiologically, clinically and anatomically as a distinct disease. They disapproved, however, of the name Amœba coli and propose the name Amœba dysenteriæ for the pathogenic form. Harris's[4] work, too, is important in showing an etiological relationship between amœbæ and a certain form of dysentery. Casagrandi and Barbagallo, in 1895, claimed that the amœbæ so far described in man show differences enough from the fresh-water amœbæ to belong to a different genus. They therefore gave the name Entamœba hominis to amœbæ of the Amœba coli type. Schaudinn (1903) was the first who based upon a definite morphology the claim that at least one species among the intestinal amœbæ is pathogenic and one non-pathogenic. The latter, which he found in normal human

[1] Hygienic Laboratory, Bull. No. 133, 1923.
[2] Kolle and Wassermann, 1913, 2d ed.
[3] Johns Hopkins Hosp. Rep., 1891, **2**, 395.
[4] Arch. Path. Anat., 1901, **166**, 67, and Am. Jour. Med. Sci., 1905.

intestines, he said, resembles those already described as Amœba coli; therefore he gave it the name Entamœba coli; while the former, which he found exclusively in ulcerative tropical dysentery, he called Entamœba histolytica.

The different views upon the relationship to disease of amœbæ found in the human intestines may be summarized as follows:

1. That the amœbæ in man have no pathogenic properties, hence are not the cause of amœbic dysentery. (Cunningham, Grassi, Celli and Fiocca, Casagrandi and Barbagallo and others.)

2. That any intestinal amœba may become pathogenic and cause the specific malady known as amœbic dysentery. (Musgrave, Clegg and others.)

3. That amœbæ are able to keep up a preëxisting inflammation. This was the original view advanced by Lösch when he described the most commonly cited form, Amœba coli, and several authors have followed Lösch in this opinion.

4. That more than one species of amœbæ are found in man, at least one pathogenic and several non-pathogenic. (Kartulis, Councilman and Lafleur, Quincke and Roos, Strong, Schaudinn, Craig and others.)

The study of bacillary dysentery by Shiga, Kruse, Flexner and others (see under Bacillary Dysentery) has demonstrated that there are at least two forms of dysentery, one produced by amœbæ and the other by bacilli, and from the work on the former it now seems certain that it is produced by one or more specific forms of amœbæ.

Amœbæ have been reported in abscesses of the jaw as well as in teeth cement and in carious teeth. Smith, Barrett, Bass and others found amœbæ in practically all cases of Rigg's disease and claimed an etiological relationship which many other authors combated.

Musgrave and Clegg[1] (1904) studied amœbæ in the Philippines by the culture method and came to the conclusion that forms obtained from various sources were probably all a single species.

Williams[2] (1911) obtained pure cultures on sterile "tissue media" of amœbæ isolated from the feces of mammals, including one from a case of amœbic dysentery.

Boeck and Drbohlav announced in 1924 that they had succeeded in cultivating Endamœba histolytica. Their full report came out in 1925.[3] Thomson and Robertson[4] corroborated this work.

Sites of Amœbæ in the Human Body.—Intestines and neighboring tissues; abdominal cavity; abscess of liver, lung, pleura, brain and mouth; necrosis of jaw-bone; tartar of teeth; urine; testes (Warthin[5].)

Materials and Methods for Study.—Human cases of dysentery showing amœbæ in stools are so seldom on hand in the northern part of this country that they cannot be counted on. The non-pathogenic form in human intestines might be obtained after administration of a saline cathartic, but generally one must depend upon entozoic forms for work with students. Material rich in saprozoic forms may be obtained from an infusion in water of lettuce, cabbage, potato skins or other vegetable material. Such an infusion should be made a week or two before it is needed, when it will be found that the pellicle which forms contains many varieties of protozoa and bacteria, among which are generally large numbers of amœboid forms.

If one has material containing human intestinal amœbæ, kittens or puppies may be fed with the cysts in order to obtain a new supply. The amœbæ should be examined in both the fresh and fixed condition. Cultures may also be made as described below.

Examination of the Fresh Material.—The study of the living amœbæ is extremely important. This may be done by making a hanging drop or hanging

[1] Amebas: Their Cultivation and Etiological Significance, Manila Bureau of Public Printing, 1904.

[2] Jour. Med. Res., 1911, **20**, 263.

[3] Am. Jour. of Hyg., 1925, **5**, 371.

[4] Jour. Trop. Med., 1925, **28**, 345.

[5] Jour. Inf. Dis., 1922, **30**, 559.

mass from fluid containing amœbæ. The size, kind of motion, frequency of pulsation of contractile vacuole, and as much of the cell contents as possible should be noted.

The stools should be examined on the warm stage as soon as possible after their passage (not later than two hours), and should be kept at blood heat until examined. A platinum loopful of material should be taken from the slimy masses in the thinner part of the feces, diluted with physiological salt solution, covered with a cover-glass, and examined under moderate magnification.

Harris[1] found that a drop of a watery solution of toluidine blue added to a small particle of the feces stains the entoplasm of the amœbæ at once and the ectoplasm a few minutes later. The amœbæ seem to be quickly killed and often when natural forms are beautifully preserved the preparations, after being washed in water and mounted in Farrant's medium, may be preserved for months, but after a time the stain completely fades.

Permanent Preparations.—Thin films are made on glass slides or cover-glasses, and immediately, before they are allowed to dry, they are placed in the fixing solution. Cover-glass may float film down, on the surface of the fixative. Among the best fixatives are: hot sublimate alcohol (50° C.), Zenker's fluid, hot Hermann's fluid, or methyl alcohol (see Stains).

Stains.—Several of the many good staining methods are given in the chapter on Stains.

1. Thin Delafield's hematoxylin, from one-half hour to several hours, then washed in water. (If overstained, the preparation may be differentiated in acid alcohol, controlling under the microscope, then washed in water.) The film or section is then passed successively through 70 to 95 per cent. and 100 per cent. alcohol, absolute alcohol + xylol, xylol, cedar oil, or Canada balsam.

2. Heidenhain's iron hematoxylin (see Stains). The smear is changed from distilled water into the iron-alum mordant for four to twelve hours, or overnight; well washed in distilled water; in stain from two to twenty-four hours, excess washed out in the iron mordant, controlled under the microscope (as decolorization occurs very quickly) until the nucleus is sharply differentiated; the chromatin of the nucleus must be a deep blue black, and the cytoplasm a light gray; then a thorough washing in tap water and passage through the alcohols and xylol, and in Canada balsam, or cedar oil for mounting.

3. After fixation in methyl alcohol one may use Giemsa's staining method (see Stains), or a modification of the method suggested by Van Gieson for staining the Negri bodies in smears.

Masses containing amœbæ, as mucous flakes or portions of the intestinal or liver abscess wall in amœbic dysentery, or pieces of decaying vegetables may be fixed *in toto* in hot sublimate alcohol for one-half hour, washed in iodin alcohol for twenty-four hours, passed through the different strength alcohols and imbedded in paraffin (see Chapter III) for section cutting if desired.

Cultures of Amœbæ. Pure mixed cultures of culturable forms may be made in the following way: from the material containing amœbæ a small loopful is removed with a platinum wire and isolated spots are touched over the surface of amœba agar (see Chapter IV) poured into sterile Petri dishes. If necessary, feces may be thinned with physiological salt solution before planting. In one to several days at 25° C. the amœbæ with the accompanying bacteria may overgrow the entire plate. We have found that amœbæ will grow as well upon nutrient agar—better with certain bacteria—as on the special medium just mentioned. Impression films may be made of these cultures, or small pieces of agar and culture may be imbedded entire. From such a culture the "pure mixed" cultures of Frösch may be made as follows: The amœbæ which have crept out to the periphery of the growth are taken out with their accompanying bacteria and transplanted. Usually one or two organisms favorable for the growth of the amœbæ accompany them, and in this way one may finally get the amœbæ growing with one definite bacterium. We have

[1] Am. Jour. Med. Sci., 1905.

isolated from a culture a single amœba unaccompanied by bacteria by the following simple method: under the low-power lens with a fine platinum loop an isolated amœba is drawn toward the edge of the agar plate. When it is well separated a disk of agar containing it is cut out following the margin of the objective and is transferred to a fresh agar plate. A very small quantity of a desired bacterium is now added to the disk near the amœba, and a "pure mixed" culture results.

Barret and Smith[1] described the cultivation of an endamœba from the intestines of a turtle.

The mixed cultures of Endamœba histolytica are made according to Boeck and Drbohlav as follows: a solid medium of either buffered Ringer's solution agar (1.4 per cent.) or of blood agar, N.N.N. medium, is used. A fluid medium is made by adding the white of 1 large sterile egg to 1 liter of the buffered Ringers' solution. This liquid is added to tubes of slanted solid medium until it is about 1 cc. above the slanted medium. This medium must be at incubator temperature (37° C.) when it is inoculated with histolytica infected material. Various modifications of this medium have been recommended.[2] Similar technic has been used to grow the other endamœbas found in the human intestines.[3]

Pure Cultures.—Certain varieties of amœbæ from the feces of mammals may grow without other organisms, *i. e.*, may grow in pure cultures, when inoculated on a piece of fresh sterile brain, kidney or liver placed upon Musgrave and Clegg's amœba agar or on nutrient agar. They grow abundantly on such media at temperatures varying from 22° C. to 38° C. (Williams).[4]

Differentiation of the Species.—The most important practical point is to be able to distinguish the forms of the species claimed to be the only pathogenic species, namely, Endamœba histolytica, from the parasitic but supposedly non-pathogenic species occurring most frequently in the feces, namely Endamœba coli and Endolimax nana. According to the descriptions given of Endolimax nana (Wenyon and O'Connor,[5] Dobell and O'Connor[6]) it is easy to differentiate. It is much smaller than the others, with an average diameter of 8μ. The nucleus is vesicular with membrane free from chromatin. The chromatin is contained in a large irregular karyosome. There is a clear ectoplasm, but no sharp line of division between it and the vacuolated finely granular endoplasm containing bacteria. It has a few blunt pseudopodia. The cysts are oval, colorless and thin-walled and finally contain four nuclei similar to those in the amœboid stage. Kofoid and Swezy[7] claim as possibly pathogenic a form distinct from others described, but heretofore included among some of the appearance ascribed to *End. coli.* They have given it the name *Councilmania lafleuri.* The differentiation claimed for End. histolytica and End. coli may be summed up as follows: (1) End. coli is, on the whole, smaller than End. histolytica; (2) its ectoplasm is so small in amount and so slightly differentiated that it is only seen when the organism puts forth pseudopods, while the cortical zone of End. histolytica is wider and is distinctly differentiated from the entoplasm; (3) the pseudopods of the former are small, rounded,

[1] Knowles: An Introduction to Medical Protozoölogy, Calcutta, Thacker, Spink & Co. 1928, pp. 648–651.
[2] Thomson and Robertson, Jour. Trop. Med. and Hyg., 1925, **28**, 345.
[3] Jour. Med. Res., 1911, **25**, 263.
[4] Am. Jour. Hyg., 1923, **3**, 205.
[5] Jour. Roy. Army Med. Corps, 1917, **28**, 557.
[6] The Intestinal Protozoa of Man, London, 1921, p. 31.
[7] Proc. Exp. Biol. and Med., 1921, **18**, 310.

delicate, and not highly refractive, those of the latter are larger, finger-shaped, firmer, and more highly refractive, thus indicating the power of the organism to penetrate its host's tissue; (4) the nucleus of End. coli is very distinct in life as well as in stained spreads, due to a definite membrane, a more distinct karyosome, and much chromatin which is distributed throughout the nucleus with more of a collection about the periphery; the nucleus of End. histolytica on the other hand, is seen with difficulty during life, and stains faintly, owing to its delicate membrane, its small amount of chromatin and small karyosome; the chromatin is collected about the karyosome and the periphery of the nucleus; the nucleus, moreover, is much more variable in shape in the active organism than is that of the End. coli; (5) the entoplasm of End. coli is less granular and vacuolated and contains fewer red blood cells than that of End. hystolytica which sometimes shows immense numbers of these blood cells (Plate IV, Fig. II, 1–5).

The above points of difference cited for organisms in the amœboid stage may hold for forms living in the human intestines; but we have found that cultivable organisms isolated from widely different sources (*e. g.*, intestines of guinea-pigs and of dogs from New York and of humans from the Philippines) when grown with a favorable bacterium in the thermostat at body temperature may show appearances similar to each other and similar also to those described for Endamœba histolytica. Most of the cultivable forms ingest red blood cells readily under favorable conditions (Williams).

Reproduction.—In the vegetative stage probably all these forms divide by a primitive mitosis, though Schaudinn, Craig,[1] and others saw only amitosis. All of our culture forms divide by mitosis, and many observers have recently reported similar division in related forms. Schaudinn and others state that End. coli in the vegetative stage may divide by breaking up (schizogony) into, at the most, eight daughter cells. In the latter instance, according to these authors, the nucleus undergoes a somewhat complicated process of division. But Walker, Hartmann and Williams have presented evidence to show that this is probably not true. The nuclei continue to divide by binary mitosis.

The vegetative stage of each intestinal organism takes place in the upper part of the intestines; as the feces become thicker most of the vegetative forms die off, while some pass on to permanent cyst formation. As with many coccidia, parasitic amœbæ may pass through a long period of vegetative life before entering upon another phase wherein forms are produced capable of infecting a new host. The length of this period depends upon a number of circumstances. Under conditions favorable for the growth of the amœbæ, as in cases of diarrhea, the vegetative phase is considerably lengthened, while in healthy intestines, as the amœbæ pass down with the thickening feces, the infecting cysts are more or less quickly formed.

[1] Jour. Inf. Dis., 1908, **5**, 324; Arch. Int. Med., 1914, **13**, 917; Jour. Inf. Dis., 1913, **13**, 30.

The two other species described as parasitic in man, but not pathogenic, namely, Iodamœba williamsi and Dientamœba fragilis, have been found very infrequently. The former is differentiated easily by the large amount of glycogen in the cysts which gives the mahogany stain with iodine ("iodine cysts," Wenyon, 1916). Dientamœba fragilis is very small and delicate, and contains in the majority of the amœboid forms two nuclei. (See Dobell for details.)

Viability.—The pathogenic amœbæ are apt to lose their motility very quickly above or below body heat, while the entozoic forms, unless grown for a long time at high temperature (38° C.), remain motile at higher or lower degrees. Though the former lose their motility, they are not all killed by cold. They may still be infective after freezing. Musgrave kept an encysted culture from a dysenteric stool at −12° C. for forty-five days and found it still viable at the end of that time.

A temperature of 60° C. for one hour usually kills encysted cultures of amœbæ according to Strong,[1] but considerable variation has been noted in the degree of temperature necessary to destroy different strains.[2]

Enemata of quinine sulphate and saturated solution of boric acid do not affect amœbæ in the intestinal canal, though a 1 to 300 solution of quinine sulphate added to the stools invariably kills them in ten minutes.

They are also destroyed in stools by weak solution of hydrogen dioxide, potassium permanganate, toluidine blue, and dilute acids.

Luttle found that 1 to 10000 hydrochloric acid and 1 to 100 silver nitrate check motility, but do not destroy parasites except after prolonged contact. Musgrave and Clegg found that in cultures treated with 1 to 2500 solution of quinine hydrochlorate the parasite quickly encysts, and in from five to eight minutes may break up and disappear; ten minutes later cultures made produce no growth of amœbæ, while the bacteria grow well. Emetin has a marked effect *in vitro* and *in vivo*.

Pathogenicity.—Lower Animals.—In regard to amœbæ from tropical dysentery (presumably End. histolytica), they have been shown to be pathogenic for young cats, dogs, and monkeys. The infection may take place in two ways: (1) By feeding material containing the cysts; (2) by rectal inoculations of the vegetative forms. The best work done on dogs is by Harris,[3] in 1901, who found that puppies were particularly susceptible after rectal injections of fresh material from human dysentery cases. Morphine was administered before the injection in order to retard peristalsis. The disease developed in two or three days and lasted from four to sixteen days.

The chief symptoms were a bloody diarrhea and progressive emaciation. The lesions observed in the intestines on postmortem examinations were a swollen and congested mucosa, over which were scattered numerous small ulcers. In two cases there were liver abscesses.

[1] Osler's Mod. Med., Philadelphia, 1907.

[2] An air-dried agar plate culture of "Amœba coli" given us by Dr. Calkins who obtained if from the Philippines was viable after three years at room temperature kept in the dark. Some of our own subcultures from this plate were alive after six years in brain medium in tubes sealed with paraffin and kept at room temperature.

[3] Am. Jour. Med. Sci., 1905.

Microscopically the mucosa first showed slight exudative and productive inflammation, followed by necrosis and desquamation of the epithelial cells and their basement membrane. At the same time the interglandular tissues beneath became swollen and small hemorrhages occurred. Great numbers of macrophages collected. Ulceration proceeded from above downward. Many amœbæ were first seen in and between the epithelial cells, then in the connective tissue at the base or sides of the ulcers. Necrotic and suppurative processes producing varying degrees of suppurative inflammation may complicate the lesions.

The abscesses which form in the liver contain degenerated liver cells, polynuclear leukocytes, red blood cells, and groups of small amœbæ.

As controls Harris tried rectal injections of various bacteria, including the Shiga bacillus. All gave negative results, however, and he considered that the amœbæ showed their specific action very plainly.

Musgrave and Clegg injected "pure mixed cultures" of material from cases of clinical amœbic dysentery as well as similar cultures of amœbæ from various sources into monkeys and produced dysentery. Musgrave fed monkeys with encysted amœbæ in bacterial cultures and obtained, in a small percentage of the cases, dysenteric stools and ulcerations in which amœbæ were found without their accompanying bacteria. Kartulis, Kruse and Pasquale, and Strong injected into the rectum the contents of liver abscesses containing apparently only the amœbæ and produced typical dysentery, with lesions similar to those seen in man.

Strong states that the lower monkeys and the orang-outang in the Philippines contract the disease naturally.

In Man.—According to an analysis made by Dobell (1921) of over 3000 individuals who have never left the British Isles, about 3 per cent. harbor End. histolytica, 18 per cent. End. coli, 5 per cent. Endolimax nana and 0.25 per cent. Iod. williamsi.

Walker and Sellards[1] carried on an extensive study of the pathogenicity of the different forms in human beings—60 Philippine prisoners—which they divided into three groups:

1. Twenty men fed with cultures of amœbæ isolated from stools or Manila water. The same amœba was recovered in cultures from several of the cases. No lasting parasitism occurred, neither did any case of dysentery result.
2. Twenty men were fed stools containing End. coli. No cultures were recovered; no cases of dysentery occurred, but 17 cases were parasitized.
3. Twenty men were fed stools containing End. histolytica. No cultures were recovered; 4 cases of dysentery occurred, and 17 cases were parasitized.

They conclude from these studies that they have furnished the proof of the pathogenicity of one form (End. histolytica), the non-pathogenic but parasitic nature of the other form (End. coli) and the saprozoic nature of cultural forms.

Amœbiasis.—The disease produced by pathogenic amœbæ in man is known as amœbic dysentery (amœbic colitis, amœbic enteritis, amœbiasis, entamœbiasis).

Incidence.—The disease occurs endemically in tropical countries. It is particularly prevalent in Egypt, India, and the Philippine Islands.

[1] Philippine Jour. Sc., Series B., 1913, 8, 253.

It occurs frequently in parts of South America and southern United States. In northern United States few cases are reported, though Patterson,[1] who in 1909 described 3 cases (without a description of the amœbæ present), and who calls attention to 15 other cases reported as endemic in New York since 1893, states that this disease is probably more widespread than is generally thought, and that if it were searched for more carefully more cases would be recognized. Patterson adds to his report a bibliography of cases reported as originating in North America. Sporadic cases are found in Russia, Germany, Austria, Italy and Greece. An occasional small epidemic may occur in the milder climates. Where it is endemic the largest number of cases occur after the heavy rains have begun in early summer. Males are more frequently attacked, because more exposed to infection. It may occur at all ages, but young adults seem most susceptible. The foreign white race seems to be more susceptible than natives. Unhygienic surroundings are generally a predisposing factor, but in the Philippines individuals of all classes are likely to be attacked who do not take continuous and extraordinary precautions in regard to their drinking water. In France since the war many cases of amœbic dysentery have been reported and the question of its control is an important one.

Symptoms.—The symptoms may be mild or severe. The disease usually runs an irregular course marked by periods of intermission and exacerbation. It may begin acutely with slight fever, griping, tenesmus and frequent stools. Occasionally, however, the onset is gradual, lasting from a few days to several weeks. The disease is generally chronic, extending over a period of a few weeks or of many years. In the mild form which is usual in children, the general conditions may be remarkably good, the only symptoms worth mentioning being the increased number of stools—2 to 6 in twenty-four hours —which contain few to many amœbæ. In the severe forms there is a loss of appetite, great emaciation, some fever, acceleration of the pulse, sweating, abdominal pains and a decided increase of the number of stools—20 to 50 daily. The stools are more fluid and slimy and may be bloody. They contain amœbæ in varying numbers. In very severe forms the stools are watery, filled with blood, mucus and sometimes sloughs. They vary in numbers from 20 to 60 in twenty-four hours and may contain many amœbæ.

The milder forms may change suddenly to the severest, and the severest may suddenly become better and completely recover.

Tissue Changes.—The lesions are chiefly in the large intestines. The walls are thickened in chronic cases, especially the submucosa. There are raised hemispheric areas of hemorrhagic catarrh and of ulceration. The whole of the large intestines may be affected or only more or less circumscribed areas. The amœbæ pass between the epithelial cells, generally through small erosions, and they finally reach the submucosa by the lymph channels. Here reproduction takes place and the irritation to the tissue causes edema and infiltration of small spheroidal cells. This produces small elevations into the lumen of the intestines. The epithelium over these raised areas is finally eroded and then bacteria and intestinal contents help form the succeeding ulcers. The erosions or ulcerations have congested undermined margins and yellowish-red bases. They vary in size from 2 mm. to about 2 cm. They are round, oval, or irregular in outline. The ulceration usually extends only to the submucosa, but may expose the peritoneum, and large sloughs may be cast off into the lumen of

[1] Am. Jour. Med. Sc., 1909, **138**, 198.

the intestines. Generally the slow inflammatory process in the submucosa leads to great thickening of the intestinal wall.

The processes may be modified in various ways by the action of other micro-organisms, especially the bacteria in the feces. Healing takes place by the formation of connective tissue in the floors and by a gradual covering over with epithelium. In extensive lesions scars may form.

Peritonitis may occur with the production of an opaque gelatinous fibrinous fluid in which the amœbæ may be found.

Abscesses may form in the liver (about 20 per cent. of all cases), less often in the lungs, and only occasionally in the brain and spleen. Amœbæ may reach the liver through lymph channels, portal vein and peritoneal cavity. The other organs are only slightly changed. Amœbic abscesses may occur in the liver or brain with no signs of dysentery, present or past.[1]

Source of Amœbæ.—If pathogenic amœbæ are strict parasites, the source would be only those substances contaminated with the host's excretions containing cysts and the "healthy carrier" would be of most danger to the community. Fresh foods and drinks of various kinds may mechanically convey these cysts. Flies may also be such conveyers. Strong states that in Manila amœbæ were cultivated from the water in large numbers in 1902, but no attempt was made to demonstrate their pathogenicity. In 1904, however, Musgrave notes the occurrence of dysentery in a monkey following the ingestion of cysts from a culture of a water amœba, though in a few experiments he was unable to infect cats from the amœbæ obtained from his monkey.

As dilute acids quickly kill the motile amœbæ, it is probable that any of those ingested in this form are destroyed in the stomach.

Immunity to the disease seems to exist. It is supposed that the amœbæ as they die produce toxic substances which call forth antibodies, but this has not yet been determined. The necrosis produced in the liver abscesses when bacteria are absent is an indication of the production of necrogenic substances.

Prognosis.—The percentage of deaths in the severe cases is quite large, especially if accompanied by abscess of the liver. Probably 25 per cent. of all cases are fatal. When treatment is begun early the prognosis is better.

Treatment.—Emetin has been found to be practically a specific curative treatment when amœbæ are in the vegetative stage, but it does not affect cysts. In the form of ipecacuanha it is highly recommended by Manson, Dock,[2] and others, especially since the introduction of salol-coated pills which allow the remedy to reach the intestines before it is absorbed, so that large doses may be given, without inducing marked nausea and vomiting. Emetin may be administered hypodermatically (Vedder, 1911). Emetine bismuthous iodide (Du Inez, 1915) has been tried by a number of investigators with more encouraging reports than from any other emetin compound.

Points in Diagnosis of Amœbæ Found in Man.—Examination of stools should be made as quickly as possible after they have been passed and they should be free from urine. The amœbæ should be seen motile because, after encystment or death, it may be difficult to distinguish

[1] Ravaut and Charpin: Presse Méd., 1919, **27**, 65.
[2] Jour. Am. Med. Assn., 1902, **4**, 15.

them from other intestinal contents. Bloody mucus or small pieces of necrotic tissue should be examined first, as they often contain large numbers of amœbæ.

If the movements are solid a dose of salts should be given and the fluid part of the resulting stools examined.

Craig differentiates living pathogenic forms from non-pathogenic varieties by the former's (1) larger size, (2) greenish color, (3) distinct hyaline, refractive ectoplasm, (4) faint nucleus, (5) many vacuoles and red blood cells, (6) marked motility.

An absolute diagnosis of liver abscesses can often only be made by an exploratory puncture and the finding of the amœbæ. If this is done the surgeon should be at hand to operate if necessary.

End. buccalis is usually found in the thick group of leukocytes and microörganisms collected between the teeth. The amœbæ are distinguished from the leukocytes and cell detritus by (1) their large size, (2) their light, highly refractive greenish appearance, (3) their glistening red color in contrast to the yellowish-red of the leukocytes when hanging drops are stained with enough of a concentrated solution of neutral red to make them appear pink.

Differential Diagnosis between Amœbic and Bacillary Dysentery.—In amœbic dysentery (1) the disease is generally chronic; (2) dysentery bacilli are usually not found in feces; (3) no severe toxic symptoms present; (4) abscess of liver frequent sequela; (5) lesion is in cecum and descending colon, not in small intestines.

In bacillary dysentery, the finding of the bacilli, and a positive agglutination test, together with the clinical symptoms of intoxication make a positive diagnosis.

AMŒBÆ IN DISEASED CONDITIONS OTHER THAN ENTERITIS.

Baelz found a very large amœba in the bloody urine and in the vagina of a twenty-three-year-old Japanese who was suffering from tuberculosis of the lung. Jurgens, Kartulis, and Posner also reported finding similar amœbæ in cases of cystitis and bloody urine. That amœbæ have been found in liver and brain abscesses without enteric symptoms has already been mentioned.

Kofoid and Swezy[1] believe that E. histolytica is the cause of the non-bacterial type of arthritis deformans.

Many studies have been made on the amœbæ present in pyorrhea alveolaris. The claim of Smith and Barrett,[2] Bass[3] and others that a specific amœba is the cause of this condition has not been substantiated. In examining many school children we[4] found that similar amœbæ in large numbers are found in the majority of cases, but no definite relation could be traced between them and the beginning of pyorrhea. That there are varieties of amœbæ in this condition, other than Endamœba gingivalis, is probable.

[1] Calif. State Med. Jour., 1922, **20**, 59; Univ. Calif. Pub. Zoöl., 1924, **26**, 165.
[2] Jour. Am. Med. Assn., 1914, **63**, 1746.
[3] Ibid., 1915, **64**, 553.
[4] Reprint No. 37, Dept. of Health, New York City, 1915.

CHAPTER XLIII.

SPOROZOA. CILIATA.

THE forms in the group sporozoa, which are parasitic in man, or which are of some medical interest, are, Coccidia, Nosema, Sarcocystis, Babesia, and Plasmodium malariæ and its allies. Babesia and the malarial organisms will be considered in separate chapters.

Only one genus among the ciliates is pathogenic for man.

The general characteristics have already been described in Part I.

EIMERIA STIEDÆ (COCCIDIUM CUNICULI).

The Coccidium cuniculi is a sporozoan parasite of the rabbit, first described by Lindemann in 1865. Young rabbits are especially susceptible, and extensive epidemics may occur in breeding houses.

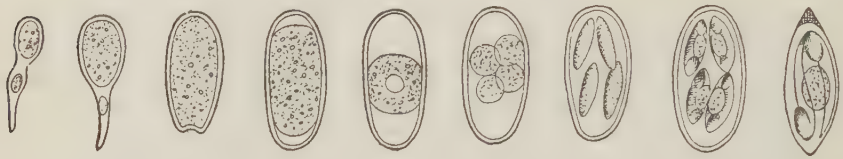
Materials and Methods for Study.—Rabbits infected with Coccidium cuniculi are often found, and the whole course of the infection may be followed with more or less ease.

A certain amount of development may be watched in hanging drops of salt solution emulsions. Sections and smears are prepared as described in Chapter III.

The cysts are stained with difficulty. It is recommended that a thin solution of Delafield's or Grenacher's hematoxylin be used for twenty-four hours followed by eosin. Heidenhain's iron-hematoxylin stain (see Stains) followed by Bordeaux red is especially good for sections.

The *symptoms* of the disease are fever, diarrhea, yellowish mucus discharge from the nose and mouth, and progressive wasting. The liver is much enlarged and shows throughout its substance variously sized grayish-white tubercles, generally surrounded by a capsule, and containing a slimy mass of degenerated host cells, in which the parasites are imbedded. The parasites are also found in the feces and in the epithelial cells of the intestines, gall ducts, and liver. The acute stage of the disease lasts about three weeks. The contents of the coccidial tumors in animals that have withstood the infection may later be emptied, leaving only a mass of cicatricial tissue. In such animals the oöcysts may remain for a long time in the gall-bladder and intestines, and by passing out gradually with the feces may provide a source of infection for other animals. The infection is carried by food soiled with cyst-containing feces. The cysts pass with the food into the stomach, where the cyst wall and the spore sac are destroyed and the sporozoites are set free. The motile sporozoites pass through the ductus choledochus into the liver, some probably passing into the intestines and infecting the cells directly, a later infection of the intestines occurring from forms developed in the liver. The organism develops within the epithelial cells of the liver and gall ducts until the cells are finally broken down and tissue cysts are formed, within which, after more or less complicated changes, cysts of the parasite are again formed. The life cycle of Eimeria Schubergi, a similar coccidium from the intestines of a myriapod, is shown on Plate IV, Fig. III, A, 1–16.

A few cases of human infection of the liver with the Coccidia have been reported. Isospora hominis Rivolta, found a few times in the human intestines, has been shown to be a different genus. Coccidiosis is so rare in man that the forms have been little studied. Some forms seem to resemble Eimeria closely, but are considered a different species from the coccidium occurring in the rabbit. Haughwout[1] described 34 cases of human coccidiosis occurring in Manila.



a *b* *c* *d* *e* *f* *g* *h* *i*

FIG. 190.—Showing spore formation in Coccidium cuniculi from the liver of the rabbit: *a* and *b*, young stage in the epithelial cells of the gall ducts (the small oval is the cell nucleus); *c*, *d*, and *e*, the fertilized oöcyst; in *d* the protoplasm is beginning to shrink away from the cyst wall, and in *e* it has contracted into a spherical form; *f*, segmentation into four sporoblasts; *g*, elongation of the sporoblasts to form spores; *h*, four complete spores in the oöcyst; *i*, single spore more highly magnified, showing the two sporozoites and a small quantity of residual protoplasm. The life cycle has been fully worked out by Simon. (After Balbiani, from Doflein.)

Isospora bigemina (Stiles) is found in the feces of dogs, cats, polecats, and possibly human beings. The organism is characterized by the division of the oöcyst into two united cysts containing four spores. The size is 8μ to 15μ. The life cycle is not well known.

Rhinosporidium kinealyi (seeberi), the name given by Minchin and Fantham (1905) to a sporozoan-like organism found in the nasal mucous membrane of certain cases from India that were troubled with hemorrhagic nasal polyps, has been shown by Ashworth[2] to be a fungus.

SARCOSPORIDIA.

This order is very little known but, considering the fact that through eating uncooked infected meat it may be found in man, though rarely, its chief characteristics should be noted here.

The Sarcosporidia are parasites of the striped muscles or connective tissue of some of the warm-blooded vertebrates (various birds and mammals). They are found in the adult state in elongated sacs known as "Rainey's" or "Miescher's tubes" (Fig. 191).

The trophozoite is a motionless elongated body, limited by a cuticle growing into a complicated structure. Spore-formation begins at an early stage and proceeds during the growth of the trophozoite (Neosporidia) which may become very large. The spores, which are many, are minute sickle-shaped or spindle-shaped mononucleate bodies with a delicate envelope and at one pole an oval striated body which represents the polar capsule found in the myxosporidia (Fig. 192).

In some cases the cyst wall calcifies and the contents of the cyst degenerate, with apparently no harm to the host; in other cases the cysts burst and their

[1] Phil. Jour. of Sc., 1919, Sec. B., **13**, 79.
[2] Trans. Roy. Soc. Edin., 1923, **53**, 301.

contents spread into the surrounding tissue, producing abscesses and tumors as with many myxosporidia, and sometimes causing the death of the host.

The symptoms of sarcosporidiosis in the pig are paralysis of the hind extremities, a skin eruption, and general systemic symptoms, such as increased temperature and pulse.

In sheep especially the disease often causes fatal epidemics. In the mouse Sarcocystis muris is a deadly parasite. Theobald Smith[1] showed that gray and white mice may become infected with Sar. muris by eating infected mouse flesh containing motile sporozoites.

Laveran and Mesnil[2] claim to have extracted a toxin (Sarcocystin) by means of glycerin or salt solution, which they have found extremely toxic for experimental animals (0.0001 gm. kills 1 kgm. of rabbit). The dried and powdered extracts are also virulent. These extracts will remain virulent for a long time in the ice-box, but will not withstand heating above 60° C. for any time.

Darling[3] (1909) describes a case of human sarcosporidiosis occurring in Panama from which he studied the organism and came to the conclusion that it was probably a different species from the one already described as occurring in man. He gives a good historical review. Later he decided that morphologically his human sarcosporidia are identical with Sarcocystis muris.

FIG. 191.—Sarcocystis tenella from the striped muscle of a swine. A full-grown cyst, showing radiately striped membrane, which is broken on the right side. (Bertram.)

FIG. 192. — Sarcocystis miescheri, *a*, small cells from a cell group; *b*, loosening of the protoplasm from the cell wall; *c*, *d*, sickle-shaped bodies (sporozoites) formed from the small cells. (From Wasielewski.) (Manz)

CILIATA.

Balantidium Coli (Malmsten, 1857). The body of this infusorium is egg-shaped with a funnel-shaped mouth opening and an average size of 60μ by 50μ. The surface of the body is covered with a pellicle, under which is a distinct ectoplasmatic sheath containing rows of basal granules from which short, fine cilia arise.

[1] Jour. Exp. Med., 1901, **6**, 1; Jour. Med. Res., 1905, **13**, 429.
[2] Compt. rend. Soc. de biol., 1899.
[3] Arch. Int. Med., 1909; Jour. Exp. Med., 1910, **12**, 19.

The cloudy entoplasm contains fat and starch granules and may contain many red blood cells and other food particles from the host. Two contractile vacuoles have been seen. Posteriorly there is a small prominence marking the place where excreta are expelled. The chromatic macronucleus is bean-shaped, and the vesicular micronucleus is nearly spherical (Plate IV, Fig. iv, A–D).

Division is transverse, the macronucleus dividing by simple constriction and the micronucleus by mitosis. Conjugation has been observed. Spherical cysts surrounded by a thick membrane are formed.

Balantidium coli has been found in the large intestines of human beings and of swine—probably two distinct varieties. The variety occurring in human beings has been found in about 154 cases, principally in Sweden, but also in Russia, Scandinavia, Finland, China, Italy, Germany, the Philippines, and the United States. Most of these cases were suffering from severe chronic intestinal catarrh, often accompanied by bloody diarrhea. A number of observers (Strong, Brooks, De Bellard,[1] and others) think the balantidium the primary cause of the catarrh with a mortality of 23 per cent., while others believe it to be a harmless inhabitant of the intestines, or at least only a secondary excitant (Opie, Malmsten, Doflein, and others).

Schaudinn described two additional species of balantidium found in the human intestines, which he called, respectively, Balantidium minutum and Nyctotherus faba, probably both non-pathogenic.

[1] Gaceta Med. d. Caracas, 1919, **26**, 27.

CHAPTER XLIV.

THE MALARIAL ORGANISMS. BABESIA.

Introduction.—The malarial organisms are a group of protozoan parasites found to be the cause of a definite group of specific infectious fevers in man, called by the somewhat misleading term malaria, a term which signifies "bad air."

They are classed as sporozoa, order hemosporidia, and are considered by the majority of observers as forming one genus, plasmodium.

So far as is known the only natural means by which the malarial organisms are transmitted to man is mosquitoes, genus Anopheles. A part of the life cycle of the organisms is carried on in these mosquitoes. Experimentally the disease has been produced by direct inoculation of infected human blood into humans. Accidentally it has been conveyed in transfused blood. The parasites develop in man within the red blood corpuscles which they finally destroy, thus producing the anemia and pigment granules peculiar to malarial fevers.

Historical Note.—The fevers caused by these organisms were recognized and studied as early as 400 B. C., but it was not until 1880 that the true nature of the dancing pigment, which had been observed long before, was determined. At that time Laveran announced that he had discovered a parasite in the blood which he claimed was the cause of the disease and he published a good description of several of the stages in the life of the organism. The public remained at first almost entirely unconvinced of the parasitic nature of these bodies. Many still believed that the bacillus described shortly before by Klebs was the cause of the fevers. Among others, Marchiafava[1] and Celli in Italy believed that Laveran's organisms represented areas of degeneration within the red blood cells, though Laveran himself demonstrated the organisms to them. When they began, however, to study the fresh tissue themselves they changed their opinion and later they published a number of valuable contributions on this subject. They gave the organism described by them the inappropriate name, Plasmodium malariæ. Laveran's researches were later confirmed by many other observers.

In 1885 Golgi showed that quartan fever depends upon a specific form of the parasite, and that the malarial paroxysm always coincides with the sporulation or segmentation of a group of parasites. Thus in a single infection with the quartan variety a paroxysm occurs every fourth day, with a triple infection on successive days, segmentation with its accompanying paroxysms occurs daily. Golgi and others soon showed that tertian fever and estivo-autumnal fevers were each due to a distinct variety of the hemamœba. These varieties are at present regarded by some as distinct genera, by others as species belonging to a single genus. Councilman first called attention to the diagnostic value of the different forms which appear in the blood.

Though it had been thought for nearly two thousand years that malaria is transmitted by insects, the question was not definitely settled until Ross, in 1896, clearly demonstrated that the hematozoa (Proteosoma præcox) of birds were transmitted by a certain species of mosquito. These investigations of Ross were soon confirmed by Grassi, Bignami and others. Manson proved (on

[1] Twentieth Century Practice, New York, 1900.

his son) that mosquitoes carried the infection to man. MacCallum's observations on the sexual form of halteridium (Hemoproteus columbæ) were a great advance, and Bignami, Grassi and others soon proved that all varieties of malarial fevers are transmitted from man to man by mosquitoes of the genus Anopheles. Grassi worked out the complete life cycle of the pernicious type (estivo-autumnal), while Schaudinn (1901) did the same for the tertian form. In later classifications the genus name Plasmodium was changed to Hæmacœba, but it is now considered that Plasmodium is the correct name.

Materials and Methods for Study.—If a case of malaria is at hand the organism may be examined alive under the microscope by allowing a cover-glass to drop gently upon a drop of fresh blood placed upon a clean glass slide. For finer differential points, however, smears should be made. which may be stained by any one of several methods, such as that of Jenner, Giemsa, or Wright. (See Stains.) Bass and Johns[1] state that by centrifugalizing at high speed defibrinated or extracted blood containing malarial organisms, the protozoa rise to the top of the cell column and by this means a mass of crescents or other forms can be obtained.

The Parasite.—Three distinct species of malarial organisms in man have been described: Plasmodium vivax (causing tertian fever), Plasmodium malariæ (causing quartan fever), and Plasmodium falciparum (Laverania malariæ, Hemameba precox), causing estivo-autumnal fever). The last species has been divided by certain authors into two varieties, a quotidian and a tertian, but it is the present opinion that only one species is definitely known. See table of the chief differences between these forms (also see Plate VII).

[1] Jour. Exp. Med., 1912, **16**, 567; Am. Jour. Trop. Dis., 1915, **3**, 298.

EXPLANATION OF PLATE VII.

Partly schematic. Drawn and rearranged by Williams, partly from Muir and Ritchie, partly from Kolle and Hetsch and partly original. Giemsa's stain.

The asexual forms show cycle of the organism in the red blood cells of the human host. They show schematically the time of fever and the day of segmentation.

Tertian type.

Fig. 1.—Segmented organism.

Fig. 2.—Young ring form in cell and a young form on surface.

Fig. 3.—Growing schizont; irregular form due to great motility; beginning pigment formation; red blood cell becoming paler.

Fig. 4.—Larger schizont with dividing nucleus. Red blood cells pale and stippled.

Fig. 5.—Nucleus divided into four clumps.

Fig. 6.—Further division of chromatin and formation of irregular rosette. Pigment finely granular in center.

Fig. 7.—Segmentation. Note eighteen merozoites (usually sixteen).

Quartan type. Shows following differences from tertian: Slightly larger, fewer segments (usually eight), and more regular. Pigment coarse. Red blood cells unaltered. Segmentation every seventy-two hours.

Estivo-autumnal type. Shows following differential points: Merozoites smaller and more numerous (thirty-two?); organism less motile with less pigment. Red blood cells smaller and greenish color (in fresh cells).

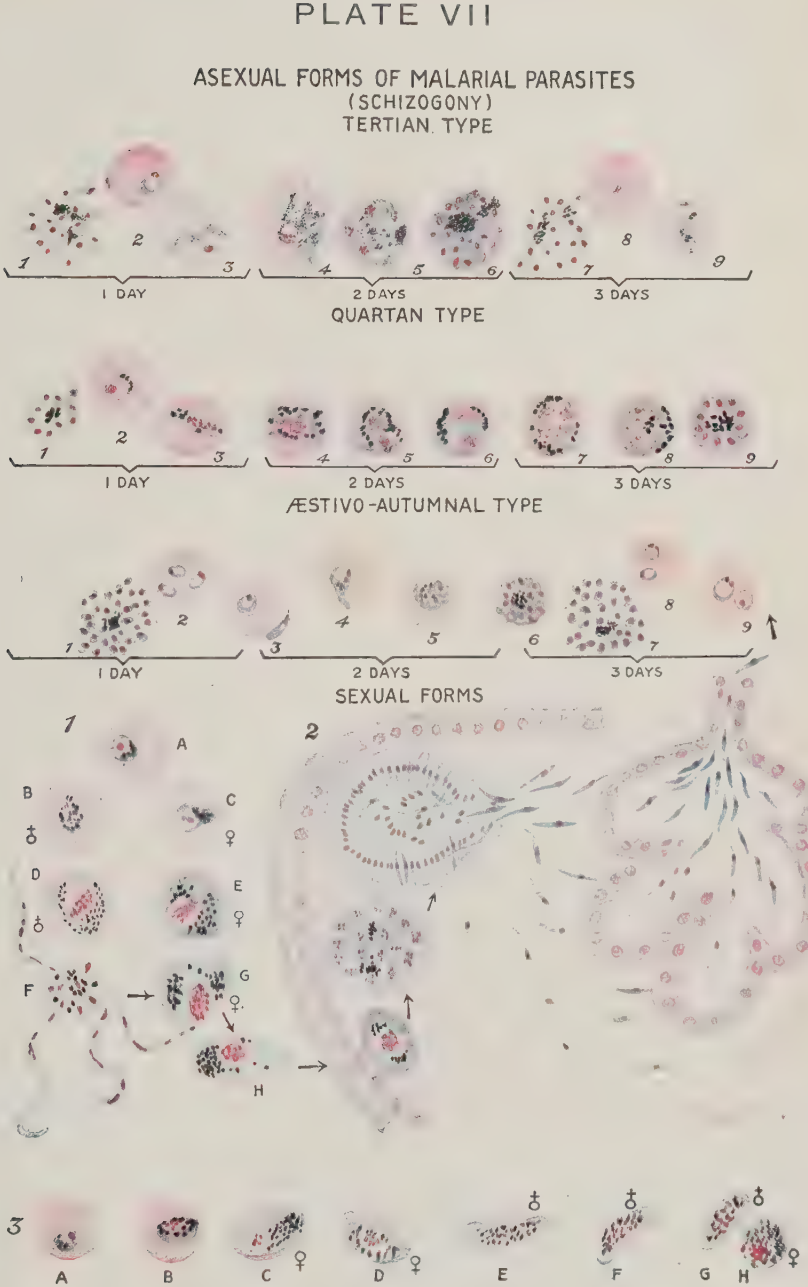
Sexual Forms. Show cycle of development in mosquito.

Fig. 1 (*A* to *E*).—Male (♂) and female (♀) forms of tertian type formed in human blood; *F*, flagellation of male type in stomach of mosquito; *G*, *H*, changes in female type and fertilization in stomach of mosquito.

Fig. 2—Development of sporocyst within mosquito. Liberation of sporozoites which find their way to the salivary gland.

Fig. 3.—Sexual forms of estivo-autumnal type found in human blood, showing development of sickle-shaped bodies.

PLATE VII



A. W. WILLIAMS, DEL.

Each of these species undergoes the two phases of development already alluded to, one within the red blood cells of human beings (the asexual phase); the other within the digestive tract of the mosquito (the sexual phase). The form changes which each parasite undergoes in humans and which the benign tertian undergoes (which may be considered a type for all) in both hosts are shown on Plate VII. Briefly, they may be described as follows:

The Asexual Cycle (Schizogony) Occurring in the Blood of Man.— The young form is often difficult to find in fresh blood. A pale area is seen on an otherwise unaltered red corpuscle, situated usually eccentrically, about one-tenth the size of the red corpuscle or about one-fourth its diameter, when at rest presenting a rounded appearance, but usually actively amœboid, throwing out distinct pseudopodia never remaining long in the same focal plane, frequently dipping, so to speak, into the substance of the corpuscle. It is often called the hyaline form because it is free from pigment, but it is not hyaline in the proper sense of the term. It is also called the ring form, because of its resemblance to a ring in stained preparations; but it is never a true ring. The ring appearance is produced by the formation of a large food vacuole. The organism continues to grow either on or in the red blood cell.

Rowley[1] claims that the organisms never enter the red blood cells. They only feed from the surface. Her beautiful demonstrations show that possibly the majority of them do this.

The forms intermediate between this and the segmentation stage appear in the fresh blood simply as larger parasites, which are readily found on account of the reddish-brown pigment granules that they contain. These granules begin to appear several[2] hours after the organism has infected the red blood cell. At this time the organism is usually actively amœboid and the granules have a lively dancing motion, due to protoplasmic currents in the parasite. The infected corpuscle is swollen and paler. In the estivo-autumnal form the infected red blood cells are smaller than normal.

When the parasite has approached nearly to its full growth, it occupies the greater portion of the corpuscle, which is now more difficult to make out. The pigment is still more evident, so that this form is therefore most readily found. At this stage amœboid movements are not so active. When full growth is reached, segmentation occurs. The forms up to the period of segmentation are called schizonts.

The morphological changes which have been going on in the parasite preparatory to segmentation are best studied in properly stained smear preparations. In the living organism they become presently sufficiently distinct to be followed; the pigment gathers more or less centrally into a compact mass, and a peripheral notching indicates that the parasite is preparing to divide into a number of segments called merozoites; the number of these segments varies in the different species. (See table.) Suddenly the segments separate as small spheroidal

[1] Jour. Exp. Med., 1914, **19**, 450.

[2] See table for number of hours in each species.

TABLE SHOWING CHIEF DIFFERENCES BETWEEN THE SPECIES OF MALARIAL ORGANISMS (GENUS PLASMODIUM) FOUND IN MAN.

Name of organism.	Size of parasite up to segmentation (schizont).	Motion of young schizont in corpuscle.	Time of appearance of melanin granules and their arrangement.	Shape of segmenting parasite, number of segments (mesozoites), and site of segmentation.	Asexual cycle complete in	Sexual forms.	Incubation period.	Effect on human host tissues.	Remarks.
P. vivax (parasite of tertian fever).	1μ to slightly larger than normal red blood cell (may occasionally be almost twice size)	Markedly active	6 hours; first scattered, then gathered in center; finely granular; actively dancing	Irregular mulberry; 12–24 (average 16); peripheral circulation	48 hours	Gametocytes spherical; no crescents; male $\frac{2}{3}$ size of female, which is $1\frac{1}{2}$ times size of red blood cell	About 14 days	Pale, granular (Schüffner's dots); slightly enlarged red blood cells; finely granular pigment formed from metamorphosed hemoglobin	Double infection may cause a paroxysm every day, thus giving clinically a quotidian type of fever.
P. malariæ (parasite of quartan fever).	1μ to little less than size of red blood cell	Not very active	Within a few hours; collected in zone on periphery; coarsely granular; slight dancing	Regular daisy in shape; 6 to 10 (average 8); peripheral circulation	72 hours	Gametocytes spherical; fewer than in vivax; about size of red blood cell; no crescents	About 3 weeks	Red blood cells may be slightly shrunken; no dots	Triple infection may cause a paroxysm every day, thus giving clinically a quotidian type of fever.
P. falciparum (parasite of estivo-autumnal or pernicious fever).	Smaller than others, from very small to $\frac{1}{2}$ diameter red corpuscle	Active; slightly less so than tertian type	Within 24 hours, small amount, 2 or 3 coarse granules usually central; non-motile or sluggish	More or less symmetrical daisy; 10 to 32 very small; chiefly in bone marrow and viscera	24 to 48 hours	Gametocytes crescentic, short and plump; $\frac{3}{4}$ size of red cell	About 10 days	Red blood cells unstained, greenish shrivelled (crenated) and darkened; stained (Giemsa) salmon color.	

bodies, the young parasites. A corpuscular remnant and the pigment float away and are ultimately ingested by phagocytic cells. The young parasites attach themselves to red corpuscles as before and the human cycle is repeated.

In a suitably stained preparation the young parasite (see Plate VII) appears to be a disk consisting of a central pale, unstained area, known as the achromatic zone, and of a basic (blue) periphery, the body, including a metachromatically stained, rounded, compact (red) chromatin mass, the nucleus, which tends to give the parasite the form of a signet ring.

Later stages up to a certain number of hours show simply changes in size and outline of the body. The nucleus then divides by simple mitosis. Later it breaks up by amitotic division into an increasing number of small masses. By the time the chromatin division is completed the chromatin masses will have assumed a rounded form, and will be seen to exhibit ultimately the same strong affinity for certain dyes which is seen in the compact chromatin body of the young ring-like form. At this stage the heretofore scattered pigment appears in one clump. Good technic will always show a corpuscular remnant even at this time. The achromatic zone mentioned will be seen to develop with the chromatin, and when the next step, namely, the division of the body of the parasite, is seen to be completed, there will be as many achromatic bodies as there are chromatin bodies, each division having a share of the basic mother-body, each representing the young parasite (merozoite).

A certain number of the full-grown parasites do not segment and these are the forms which commence the life cycle in the mosquito. These forms grow to produce the sexual forms, the macrogametocyte, or female organism, and the microgametocyte, or male organism. When mature these forms are generally larger than the mature schizont of the same species, the female organism being usually larger than the male and containing more food granules and a smaller nucleus. In the estivo-autumnal forms they are crescentic in shape, while in the other species they are spherical. In the circulating blood of human beings they show no further changes except to become freed from the corpuscle; but when the blood containing them is withdrawn and exposed for a short time to the air, an interesting series of changes in the microgametocyte is observed. The crescentic bodies are transformed into spherical bodies; the pigment of the microgametocytes becomes actively motile, due to internal agitation of the chromatin fibrils, which presently emerge as flagella-like appendages. Their movements are very rapid, causing corpuscles to be knocked about, and finally they become detached as the microgametes, or male elements. These become attached to the female element. In the withdrawn blood of birds, one may actually observe the process of conjugation in slide preparations even without the aid of a moist chamber and heat. This transformation of male bodies has not been observed in the human blood. It will be seen that it belongs to the sexual cycle which occurs in the stomach of the mosquito.

The Sexual Cycle (Sporogony) Developing in the Mosquito.—The common mosquito, often day-flying, belongs to the genus Culex; it cannot carry human malaria. It is easily distinguished from its night-flying or dusk-flying relatives, Anopheles (the malaria-carrying mosquitoes comprise about eight genera of the subfamily anophelinæ), by its assuming a different posture on the perpendicular wall. While the Culex holds the body more or less parallel with the surface, the body of the Anopheles stands off at a marked angle. Other differential points are the following (see Figs. 193–202):

Wings of Culex are unspotted; those of Anopheles are spotted (except in one rare species).

The proboscis of Anopheles points toward the resting surface, while that of Culex does not do so.

Anopheles species bite usually in the early evening, while those of Culex bite almost at any hour of the day.

The male mosquito is readily told from the female by its plumed antennæ, those of the female being inconspicuous.

The eggs and the larvæ of the two genera are quite distinct, as may be readily seen by glancing at Figs. 193 and 194. The anopheles mosquitoes breed in practically any kind of collection of water, though some species prefer slow-running water to quiet pools. The best known domestic carriers are usually found in barrels and cisterns.

If an ordinary mosquito (Culex) is allowed to imbibe the blood of a malarial patient whose blood shows gametocytes there will be simply a digestion of such blood in the mosquito, and no development of the malarial organisms results. If, however, certain species of Anopheles ingest such blood, immediate changes follow. It should be remembered that only female mosquitoes are blood sucking; hence they alone can be responsible for the spreading of the disease. It should also be remembered that if the blood imbibed by the anopheles does not contain gametocytes, though it may contain earlier stages of the malarial organisms, no amount of such blood can cause general infection of the mosquito. The sexual cycle is similar in all species of the parasite.

The flagellation of the male parasite described above will promptly take place in the stomach of the anopheles, 4 to 8 microgametes being formed; these conjugate with the female element (Plate VII) in a manner comparable to the impregnation of the ovum of higher animals by spermatozoids. The macrogametocyte becomes a macrogamete by the formation of a reduction nucleus which is thrown out of the organism.

The product of conjugation, the oökinet (zygote), remains for a number of hours in the juices of the chyme stomach, changing gradually from a spherical, immobile body into an elongated motile wormlet (Plate VII, Sexual Forms, 1 H). This penetrates the epithelial lining of the stomach and rests in the tunica elasticomuscularis (Plate VII, Sexual Forms, Fig. 2); here it changes into an oval then into a round body, which grows in the course of the next few days enormously, forming a cyst which projects into the body cavity. Meanwhile the

Fig. 193

Fig. 194

Fig. 195

Fig. 196

Fig. 197

Fig. 198

Fig. 199

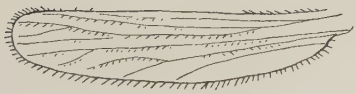


Fig. 200

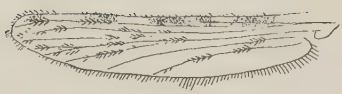


Fig. 201

Fig. 202

Chief comparative characteristics of Culex and Anopheles. (From Kolle and Hetsch.) Egg of Culex, Fig. 193, laid together in "small boat," those of Anopheles, Fig. 194, separate and rounded. Larva of *C.*, Fig. 195, hangs nearly at right angles to water surface, those of *A.*, Fig. 196, are parallel to surface. Body of *C.*, Fig. 197 when resting is held parallel to wall in a curved position, that of *A.*, Fig. 198, stands at an angle of about 45° and is straight; wings of *C.*, Fig. 199, are generally not spotted, those of *A.*, Fig. 200, are spotted. In *C.* the palpæ, Fig. 201, of the female are very short, of the male are longer than the proboscis; in *A.*, Fig. 202 the proboscides of both sexes are about of equal length.

chromatin will have become very active. It will have divided into numerous nuclei, which become arranged around inactive portions, and filamentous sporozoites develop from this chromatin and surrounding protoplasm (Plate VII, Sexual Forms, Fig. 2). These sporozoites ultimately fill the cysts, which rupture, setting them free into the cavity of the mosquito's body; they then are carried by the lymph to all parts of the body of the mosquito and thus reach a glandular structure in the thoracic cavity of the insect, the so-called salivary gland (poison gland), in which they accumulate in large numbers. This gland is in immediate connection with the biting and sucking apparatus. If, now, such an infected mosquito "bites" a human being, the lubricating fluid of the puncturing apparatus will carry sporozoites into the latter's blood and the human cycle begins. The stages of development in the mosquito require from seven to ten days, but only when the temperature is favorable.

Cultivation.—Bass and Johns[1] announced in 1911 that they had succeeded, by a rather complicated method, in obtaining a certain amount of development of P. vivax in the test-tube (see special media for details of method). Essentially, defibrinated malarial blood is used, to which is added ½ per cent. dextrose. Bass and Johns state that the layer of serum about the sedimented red blood cells must not be too deep (½ inch) and that the leukocytes must be removed if more than one generation of the parasite is wished. The Thompsons[2] (1913) claimed that they obtained several generations by less attention to these details.

They draw the malarial blood into the sterile test-tube (10 c.c.) in which there is a thick wire, and 0.1 c.c. of a 50 per cent. aqueous solution of glucose. The blood is defibrinated by gently stirring the wire for five minutes. The wire and clot are removed, and the blood is poured into smaller tubes, 1 inch in each. Rubber caps are placed over cotton plugs. These tubes are kept at 37° to 44° C. The corpuscles settle slowly, leaving about ½ inch clear serum. They have found it unnecessary to remove the leukocytes by centrifuging. They state that the malarial organisms are not destroyed by the leukocytes in the tube but by the changes in the serum. Their organisms grow through the whole depth of the layer of red blood cells.

Sinton[3] reports a simplified method of cultivating P. falciparum.

Effect on Man (Pathogenesis).—As the organism grows at the expense of the red blood cells the principal change is in the blood. Melanemia, or the formation of pigment granules from the destroyed red blood cells, is one of the most characteristic features of malaria. As the disease progresses the red corpuscles show varying changes in form and hemoglobin content, not only the infected corpuscles, but others as well, thus showing that the organism produces either primarily or secondarily some toxic substances. The pigment occurs in two forms, melanin and hemosiderin. The second only gives the reaction for iron and is found in the internal organs, while the first is found everywhere in the circulating blood. The pigment is taken up by the leukocytes. There is usually a definite reduction of both red and white blood corpuscles, which is more marked in tertian and quartan malaria than in estivo-autumnal. There is a relative increase in the number of mononuclear leukocytes.

[1] Jour. Exp. Med., 1912, **16**, 567; Am. Jour. Trop. Med., 1915, **3**, 298.
[2] Ann. Par. and Trop. Med., 1913, **7**, 509.
[3] Indian Jour. Med. Res., 1922, **10**, 203, 210.

The spleen shows marked hyperplastic inflammation and pigmentation. In intense estivo-autumnal cases the capillaries of the brain and other organs may be filled with the parasites. We have observed parasites also in the large nerve cells of the brain.

Toxin Production.—The relationship between segmentation and paroxysm is always noted in tertian cases, and it is reasonable to suppose that the occurrence of the paroxysm is referable entirely to the liberation of toxic substances resulting from metabolic activity of the parasite within the corpuscle. That there should be a toxic product seems highly probable, and its amount must be considered in heavy infections. Cases showing an infection of 1 to 5 per cent. of all corpuscles are not infrequent; the destruction of from 50,000 to 200,000 or more corpuscles per cubic millimeter of blood leads to the rapid deglobularization of the blood; hence the deficiency in numbers; add to this the effects of the metabolic products, and little is left to the imagination to explain the pronounced anemia.

Immunity from malaria appears to exist as natural and acquired immunity.

Prophylaxis.—The fact that, with the extermination of the malaria-carrying mosquitoes, malarial fevers in man would be made impossible, remains established; the parasite must have its chance of rejuvenescence in the mosquito's stomach.

The various methods of extermination are fully described in books which go minutely into the subject. The method of giving small doses of quinine to human beings exposed to *Anopheles*, and of thus getting rid of the organism itself within man, of disinfecting man, should be considered. In hot climates especially, where it is practically impossible totally to destroy the breeding places of the mosquitoes by drainage or oiling, this method is especially serviceable. Bass[1] and others have reported extensive efforts in this direction. In these countries, too, the use of adequate screening is of marked value.

Points of Diagnosis.—By a study of the parasite taken from the circulating blood the examiner should be able to tell not only the species present, but also the progress the disease is making. Malarial parasites can always readily be found in recent primary infections, and it is usually only in old cases that the search becomes difficult; one is, however, generally rewarded by finding them if one looks long enough for them.

A helpful sign is the finding of pigment in mononuclear leukocytes which are seen about the time of a chill or of the period symptomatically corresponding to it. Free pigment cannot be used as a means of diagnosis, as it may be impossible to tell it from dirt or dust. In a primary infection of *long standing* the gametocytes may be found, and in relapses and in those cases treated by quinine, many atypical forms appear. A small dose of quinine may drive all parasites except the sexual forms out of the peripheral circulation; at all events the finding of them becomes,

[1] Jour. Am. Med. Assn., 1919, 73, 21.

in the absence of gametocytes, a matter of time and experience, especially also as they may be much altered in appearance. The part most and first affected is the blue-staining body; later follow eccentricities of the chromatin, such as multiple bodies and dwarfing—just such changes as might have occurred in time, if the body had been allowed to combat the parasite without the aid of drugs. In both cases the fever curve becomes atypical. It should be remembered that there is no quotidian form originating in this country. Quotidian paroxysms occurring here are either a double tertian, or a triple quartan infection. The notion that the parasites can be found only at the time of the paroxysm is still in the minds of many; it is erroneous. The gametocytes are quite resistant to quinine and other drugs, and it appears as if cases in which these forms are seen are much more prone to relapse than promptly treated recent primary infections. The macrogametocytes may remain quiescent for years in the blood, and then under certain conditions, probably through parthenogenesis, may again begin to develop and multiply, thus bringing about relapses.

In the estivo-autumnal forms the crescentic gametocytes are generally few, but at times large numbers of them develop. Of course they are absolutely characteristic. The young parasites are more or less

DESCRIPTION OF PLATE VIII.

(After Goldhorn.)

1. Typical young tertian form; the corpuscle shows incipient degeneration; corpuscle to left above shows a blood platelet.

2. Abnormal young form, showing small accessory chromatin body.

3. Two parasites; one normal young form; the second a large form in crenated corpuscle is an unusual abnormal form with very large achromatic area.

4, 5, 6. Estivo-autumnal parasites; single, double, and triple infection; central elongated chromatin bodies. These forms are about the largest usually seen in the peripheral blood; no degeneration of corpuscle.

7. Tertian parasite, about ten hours old; marked degeneration of corpuscle.

8. Double infection of a corpuscle in tertian fever; marked degeneration of corpuscle.

9, 10, 11. Large tertian parasites showing division of chromatin previous to segmentation.

12 and 14. Complete segmentation of tertian parasite.

13. Double infection of corpuscle, one parasite reaching maturity, but showing unusually small segments; the second one atrophied.

15. Tertian parasite, old case; while the parasite is only half-grown, the chromatin has split into several compact masses. Degeneration of infected corpuscle.

16. Dwarfed tertian parasite, smaller than a red corpuscle, but showing five compact chromatin bodies; resemblance to quartan rosette.

17. Microgametocyte of tertian malaria; prominence of blackish pigment surrounding a large acromatic zone in which the microgametes lie coiled up.

18. Tertian macrogametocyte.

19 to 23. Crescentic bodies of estivo-autumnal malaria.

19. Typical gametocyte; pigment surrounding achromatic area; no chromatin shown; the "bib" is present. (Male?)

20. Semiovoid gametocyte. (Female?)

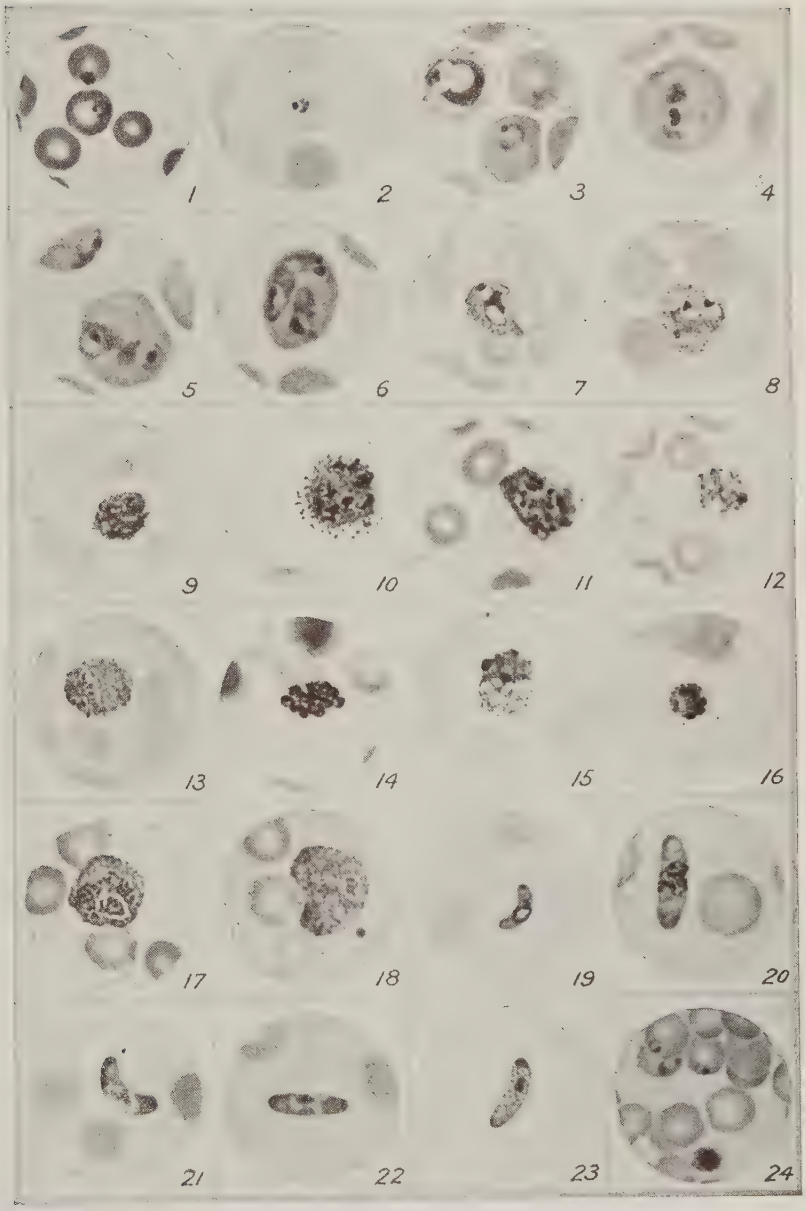
21. Pigment removed. Elliptical achromatic area in which the microgametes are seen.

22 and 23. Pigment removed; chromatin more compact; possibly female elements.

24. From a case of pernicious malaria with rich infection; only hyaline forms in peripheral blood. Below, a large blood platelet.

NOTE.—As the amplification is not uniform, a comparison of the parasites with the blood corpuscles shown should be made in order to have a correct conception of their size.

PLATE VIII



Photographs of Tertian and Estivo-autumnal Malarial Parasites in Different Stages of Development. (Goldhorn.)

characteristic in stained preparations (Plate VII). There may be as many as seven parasites in one corpuscle. Later the few heavy pigment granules are characteristic.

In fatal cases the formation of crescents may not take place; the blood infection with young parasites is then enormous, every field of the microscope showing numbers of them.

In the study of estivo-autumnal fever, as well as in that of the other forms, it is to be remembered that crescents when found indicate that the disease is of some standing, for such sexual forms are not formed until the asexual propagation is waning. The recognition of these ovoidal and crescentic bodies is easy. But as there are no readily discoverable pigmented forms in the peripheral blood in the early stages, it is necessary to be thoroughly familiar with the young estivo-autumnal forms. Polychrome staining for them cannot be too much recommended, as there is little that is characteristic about them when they have been stained with methylene blue alone. Many a serious error has been made by adhering to the antiquated idea that parasites should be looked for in the fresh blood, as these young, non-pigmented, so-called hyaline forms cannot be readily recognized by the inexperienced, while it is an easy matter to know and classify them when properly stained.

The recognition of the quartan parasite in its early stages in the fresh blood is not as difficult as that of the tertian form, because the outline is more distinct; but in stained preparations it is often indistinguishable from the latter. The living amœboid young form or schizont is more refractive than the young living tertian schizont, more like the estivo-autumnal form, and it is just as sluggish in its movements. Then, too, the corpuscle is often shrunken and looks as if it contained more hemoglobin than in the case of infection with the tertian parasite.

The growing parasite rapidly becomes pigmented, but it shows fewer, larger, less motile pigment granules than the corresponding tertian one; moreover, the pigment is arranged around the periphery of the organism, while in the tertian form it is distributed throughout the protoplasm. The quartan parasite is apt to form a band across the infected corpuscle. Segments are few in number, as a rule, and the parasite remains dwarfed while the infected red blood cells are normal in size. The segments are generally arranged symmetrically around the central pigment, giving the so-called daisy or marguerite appearance to the parasite at this stage (Plate VII).

In tertian fever the granular degeneration which the infected corpuscles early undergo is diagnostic. In the first few hours it resembles the ordinary granular stroma degeneration with basic affinity, while it is later seen that the affinity of the then more numerous granules is more acid, or, at least, the staining is no longer orthochromatic, the blue being superimposed by a red; in other words, these granules stain later metachromatically. The greater the loss or transformation of the hemoglobin the greater the number of granules. This holds good only for tertian parasites, the estivo-autumnal variety causing practically no appreciable change, though the same technic is used.

Malarial-like Parasites in Other Animals.—Hemosporidia closely related to the malarial organisms have been found in birds: the Proteosoma (Plasmodium præcox) and the Hemoproteus. Points in the life history have been brought out by various observers, especially by Ross and by MacCallum.

Malarial-like organisms have been found also in monkeys, cattle, dogs and frogs, but they have been little studied.

Blackwater Fever.—This is a condition which occurs frequently, especially in Europeans in tropical countries. Its etiology has been the subject of much discussion. The chief symptoms are fever, hemoglobinuria, delirium and collapse. It frequently ends in coma and death. A few consider it a disease entity, but the majority of observers are inclined to believe it the terminal stage of a severe malarial infection. It is frequently associated with the demonstration of the malarial organisms, but they cannot be found in some cases.

GENUS BABESIA (PIROPLASMA).

It was not until 1888 that there was a hint as to the real nature of the actual cause of "Texas fever" (bovine malaria, tick fever, hemoglobinuria) and allied diseases which attack field cattle in many parts of the world. Then Babes described inclusions in red blood cells in Roumanian cattle sick with the disease, though he did not decide upon the nature of the organism. No new studies were reported until 1893, when Theobald Smith and Kilborne[1] gave such a complete description of this disease and its cause as occurring in Texas cattle that little concerning it has since been discovered.

These authors describe as the cause of Texas fever, pigment-free amœboid parasites appearing in various forms within the red blood cells of infected animals. The organisms may be irregularly round and lie singly or they may be in pear-shaped twos, united by a fine line of protoplasm.

Because of these double pear-shaped forms Smith and Kilborne named the organism Pyrosoma bigeminum[2] and they placed it provisionally among the hemosporidia. These authors also showed that the contagion was carried by a tick (see below). Their work has been corroborated by many investigators in different parts of the world. Hartmann places this genus in his order Binucleata, and he considers it an important form for showing the relationship of the endocellular blood parasites to the flagellates. Schaudinn, in 1904, was the first to call attention to the occurrence of nuclear dimorphism in B. canis and bovis, and Luhe, Nuttall and Graham-Smith,[3] Breinl and Hindle, and others have confirmed this observation. The second nuclear mass is generally in the form of a small granule similar to the blepharoplast of undoubted flagellates.

[1] U. S. Dept. of Agri., 1893, Bull. No. 1.

[2] The generic name Pyrosoma, already in use for a well-known Ascidian genus, was altered to Piroplasma by Patton in 1895. In the meantime Starcovici (1893) had given the name Babesia bovis to the form described by Babes; and as this form seems to be identical with that described by Smith and Kilborne the name of the genus should be Babesia, but the majority of protozoölogists still call it Piroplasma.

[3] Jour. Hygiene, 1905, **5**, 485; 1906, **6**, 586; 1917, **7**, 232; also Parasitology, 1909, **2**, 215, 229, 236.

Morphology of the Parasite.—In the examination under 1000 diameters of fresh blood of sick cattle, according to Smith and Kilborne, are seen, in the red blood cells, double pear-shaped forms and single rounded or more or less irregular forms. The size varies, though generally it is the same among the bodies in the same red blood cell. The average size is 2μ to 4μ long and $1\frac{1}{2}\mu$ to 2μ wide. The pointed ends of the double form are in apposition and generally touch, though in unstained specimens a connection between them cannot be seen. The axis forms either a straight line or an angle. The protoplasm has a pale, non-granular appearance, and is sharply separated from the protoplasm of the including red blood cell. The small forms are generally fully homogeneous, whereas the larger ones often contain in the rounded ends a large rounded body, 0.1μ to 0.2μ in size, which is very glistening and takes a darker stain. Within the largest forms in the center of the thick end is a large round or oval body, 0.5μ to 1μ, which sometimes shows amœboid motions. Piana and Galli-Valerio (1895 and 1896) and other observers have since described definite amœboid motion of the whole parasite. The motion of the whole varasite on the warm stage is not produced by the formation of distinct pseudopodia, but by a constant change of the boundary. The changes can succeed each other so quickly that it is scarcely possible to follow them with the eye. The motion may persist for hours. The single ones show motion, while the double ones remain unchanged.

The parasites take most basic anilin stains well. The Romanowsky method or its modifications give the best results (Plate IV, Fig. III, B). Stained by this method the smallest forms appear as tiny rings, about one-sixth the diameter of the red blood cell. A part of the rim takes the red nuclear stain, the rest is blue. In the large mature pear-shaped organisms a loose mass of chromatin is at the rounded end and a dense, compact mass is situated nearer the pointed end. These mature, pear-shaped forms, Nuttall states, are the mark of distinction between *Piroplasma* (Babesia) and other intracorpuscular blood parasites. These pyriform bodies are generally present in pairs, and occasionally, in the acute form of the disease, sixteen pairs may be seen in a single blood cell.

The number of red cells infected is about 1 per cent. of the whole. If the number increases to 5 per cent. or 10 per cent., it generally means the death of the animal. The parasites quickly disappear from the blood after the disappearance of the fever. In fatal cases many parasites are found in the red blood cells of the internal organs. They vary in number according to the stage at which death occurs, are most abundant in the kidneys (50 to 80 per cent. of all red corpuscles infected), and are found in fewer numbers in the liver, spleen and other internal organs.

Flagella-like appendages in Babesia have been described by several observers as occurring in the blood in mammals. More frequently they have been seen in the tick and in attempted cultures. They have been interpreted by some (Hartmann, Calkins) as possible microgametes, by others (Breinl and Hindle) as true flagella, and by others (most observers) as fine pseudopodia.

Transmission.—Smith and Kilborne show that the infection here is caused by a species of tick, *Margaropus annulatus*, Say (*Boöphilus bovis*)

(Fig. 203). Kossel gives *Ixodes redivius* as the tick causing transmission of the germ in the hemoglobinuria of Finland cattle. Other ticks have been named as transmitters of the disease as it occurs in other parts of the world and in other kinds of animals.

The ticks feeding upon the blood of cattle and other mammals become sexually mature at their last moult. They then pair, and the fertilized females, after gorging themselves with the blood of their host, drop to the ground. Each female then lays about 2000 eggs, and within the shell of each egg a large quantity of blood is deposited to serve as food for the developing embryo. The female then shrivels up, becoming a lifeless skin. The newly hatched larvæ containing in their abdomens some of the mother-blood, crawl about until they either die from starvation or have the opportunity of passing to the skin of a fresh host. If the mother-tick has drawn its supply of blood from cattle infected with piroplasma, her larvæ are born infected with

Fig. 203.—No. *1*, Texas fever tick, Margaropus annulatus (Boöphilus bovis). × 15.4. No. *2*, natural size. (Mohler.)

the parasite and become the means of disseminating the disease further. This mode of dissemination explains the long incubation period of the disease (forty-five to sixty days—thirty days for the development of the larvæ and the remainder for the development of the parasite within the host). It is possible that the tick embryo acquires the infection secondarily from the blood it absorbs in the egg, and that the parasites do not pass through the ovum itself as in *Nosema bombycis*. This species of tick, *M. annulatus*, has been found also on sheep and ponies.

So far it has not been possible experimentally to inoculate animals other than cattle with these parasites. Calves withstand the infection better than older animals and a certain degree of immunity is reached in some of the older cattle in infected districts. The piroplasmata taken in by such animals may remain as harmless parasites for some time. If, however, such cattle are weakened from any cause,

their resistance to the organism may be lowered and they may therefore pass through a more or less severe attack of the disease.

Three species have been described as occurring in cattle, and other species have been described for several other animals.

Nuttall and Graham-Smith report a study of canine piroplasmosis, and have drawn a cycle showing the usual mode of multiplication in the circulating blood. They consider B. canis a species distinct from B. bovis and B. pitheci (found by Ross, in 1905, in blood of a species of cercopithecus).

Theiler showed that the protozoa causing hemoglobinuria fever in East Africa did not protect against Babesia infection, so his organism is considered a different genus, and is called Theileria parva. Transmission is by Rhipicephalus ticks.

Christophers has described probable sexual stages of development in the tick R. sanguineus, so that he has drawn a complete life cycle of the organism.

Symptoms of the Disease.—Fever (40° to 42° C.), anorexia, weakness, increased pulse and respiration, decreased secretion of milk, hemoglobinuria at the height of the fever, causing the urine to appear dark red like port wine or darker. The urine may contain albumin even if the hemoglobinuria is absent, but there are no red blood cells present, the color being due to the coloring matter of the blood only. There is icterus of the mucous membranes if much blood is destroyed.

The prognosis varies in different epidemics from 20 to 60 per cent. Death may occur in three to five days after first symptoms appear. Recovery is indicated by a gradual fall of the fever.

Treatment.—Quinine in large doses seems to have helped in some epidemics. Nuttall, Graham-Smith, and Hadwen have reported curative effects from trypanblue in both canine and bovine babesiosis (Piroplamosis), other than Theileria infections.

Prophylaxis.—Stalled cattle are not infected, but it is impracticable to keep large herds of cattle stalled. If the cattle are kept from infected fields for one or two years and other animals (horses and mules) are allowed to feed there the ticks may disappear. The burning of the field for one season may have a good effect. If animals cannot be taken from infected fields such fields should be enclosed.

Ticks on animals may be killed by allowing the cattle to pass through an oil bath (paraffin, cottonseed oil, etc.), whereupon the ticks die from suffocation. The bath should be repeated after a week in order to kill any larvæ which may have developed. All animals sent from infected regions should receive this treatment. Animals apparently healthy before the treatment, after the disturbing influence of the bath often develop the disease in an acute form and die.

Certain birds in Australia seem to feed on the ticks, therefore such birds might be propagated.

Various attempts have been made to give protection by the inoculation of fresh (not older than two or three days) blood from slightly infected animals. Some partial results have been reported, especially when the inoculations were made during the cold months. In Australia the inoculation of defibrinated blood from animals which have just recovered from the infection, but whose blood still contains some parasites, has been tried. So far no absolute protection has been produced, neither does the parasite-free serum of animals which have entirely recovered from the disease seem to contain protective qualities.

Cultivation.—Thompson and Fantham[1] reported development in the test-tube after the method of Bass and Johns for malaria.

[1] Ann. Par. and Trop. Med., 1913, **7**, 621.

Other Blood Organisms.—Blood organisms similar to those described in the hemoglobinuria of cattle have been found in cases of red water fever of cattle in England. They also occur in monkeys, dogs, sheep, horses and pigeons. Nocard and Motas, who have made an extensive study of these parasites in the malignant jaundice (hemoglobinuria, malaria, or biliary fever) of dogs, state that though the parasites are morphologically similar to those infecting cattle, yet it is impossible to infect cattle or any other animal tried with them. They must therefore be considered physiological species.

Strong[1] and his collaborators reported that in an extensive study of *oroya fever*, a tropical disease, they had determined the cause to be a Babesia-like organism which they called *Bartonia bacilliformis*.

Noguchi and Battistani[2] reported the cultivation of a pleomorphic microbe on semisolid medium. Inoculated into rhesus monkeys the cultures produced vascular nodules and occasionally invaded red blood cells. Noguchi's investigation on this condition and the related disease, verruga peruana, were reported in a series of articles.[2] This work has not yet been corroborated.

[1] Jour. Am. Med. Assn., 1913, **61**, 1713; 1915, **64**, 805 and 965.
[2] Jour. Exper. Med., 1926, **43**, 851; 1927, **45**, 437, 455, 781; **47**, 219, 235, 821; **48**, 619.

CHAPTER XLV.

SMALLPOX (VARIOLA) AND ALLIED DISEASES.

Introduction.—The diseases smallpox, cow-pox vaccinia, horse-pox and sheep-pox if not identical are closely allied. Indeed the following facts seem to prove that at least cow-pox and variola are very closely related if not essentially the same disease. Monkeys have been successfully immunized against either disease by previous successful inoculation of the other. Human beings who have had vaccinia through inoculation with cow-pox virus and those who have within a reasonable time passed through an attack of smallpox cannot be successfully inoculated with cow-pox vaccine. It would seem that on account of the above close relationship between the two diseases that smallpox virus when inoculated into calves should produce an infection exactly similar to cow-pox. But the successful inoculation of calves with smallpox material from human beings is a matter of great difficulty. In our laboratory not one of many attempts to accomplish it has been successful. There are, however, some experimenters who have asserted that they have succeeded. This indicates the possibility of such a transfer of the virus. All the evidence indicates that the two diseases are produced by organisms originally similar, one being modified by its passage through humans and the other through cattle.

Variola is perhaps the most regularly characteristic of the diseases of man. It is highly infectious and is controlled only by vaccination. Notwithstanding the fact that we know definitely the exact site of the infective agent in this disease and that certain experimental animals are susceptible to inoculation of the material containing the infective agent, investigators are still undecided in regard to the nature of the chief exciting factor. A few, however, think that certain bodies found chiefly in the epithelial cells of the skin and mucous membranes in the specific lesions may be protozoa causing the disease.

Historical Note.—The first undoubted description of the disease smallpox was given by Rhazes in the tenth century, but it is evident that he did not consider it a new disease.

Carried by the intercommunication, principally of war and commerce, the disease was widespread when Edward Jenner showed conclusively in 1798 that vaccination with cow-pox afforded protection. Now the cases of variola that occur are seen in those who, through neglect or ignorance (sometimes wilful), have not been vaccinated.

Etiology of Variola and Cow-pox.—It has been repeatedly shown that no bacteria similar to any of the known forms have a causal relation to these diseases. In our own laboratory we are able,[1] by the

[1] Williams and Flournoy, N. Y. Univ. Bull. of Med. Sci., 1902, vol. 2.

inoculating of rabbits' skins, to produce extremely active vaccine virus in large quantities, absolutely free from microörganisms which grow under the conditions of our present methods of bacterial cultivation. Such pure active vaccine, when emulsified in equal parts of glycerin and water and filtered through two or three thicknesses of the finest filter paper, gives a slightly opalescent filtrate, which in the hanging drop under high magnification shows many very tiny granules with an occasional large one, and in smears shows no formed elements giving characteristic stains. This filtrate, from which no growth was obtained on artificial culture media, when rubbed over a freshly shaved rabbit's skin after the method of Calmette and Guérin, or when used to vaccinate human beings, gives an abundant typical reaction.

These facts show that some, at least, of the infective forms cannot as yet be made to grow outside of the body, that such forms are very minute, and that they do not stain characteristically with our usual methods of staining. In 1905 we had one successful result out of many with filtered material, but the controls were not sufficient to rule out accidental infection. Since then Bertarelli and a few others have reported that the virus is slightly filtrable under pressure. Gurley in our laboratory found that by first centrifuging the virus emulsion and then filtering under 15-pounds' pressure she was able to get a slight infection with the filtrates.

Steinhardt, Israeli, and Lambert[1] (1913) report that evidence of multiplication of germs may be obtained by growing the virus on living tissue *in vitro*.

Since Guarniéri, in 1892, claimed that certain inclusions present in the epithelial cells of the lesions of smallpox in a rabbit's cornea (Fig. 204) were parasites, much attention has been given to the study of these bodies, commonly known as "vaccine bodies," yet opinions still differ as to their nature. Among the more important studies of these bodies are those, on the one hand, by Councilman[2] and his associates who believe them to be protozoa, and, on the other, by Ewing,[3] who believes that all of the forms so far described are degeneration products, some specific, others not.

Calkins, working with Councilman, thinks that his original tentative cycle is too elaborate. He still firmly believes that the bodies are protozoa, but that they belong among the rhizopoda and not among the microsporidia where he first placed them.

Prowazek and others stated that the organisms of this group of diseases, as well as of rabies, trachoma, and a few others, are all minute coccus-like forms which have the power of producing an envelope from the host cell substance, such envelope with its contained organism constituting the specific body which others have called a protozoön. Prowazek[4] calls the group Chlamydozoa and says they probably stand between the bacteria and the protozoa in systematic

[1] Jour. Inf. Dis., 1913, **13**, 294; 1914, **14**, 87.
[2] Jour. Med. Res., 1904, **12**, 1; Osler's Modern Med., Philadelphia, 1907, vol. **2**.
[3] Ibid., 1904, **12**, 509.
[4] Deutsch. med. Wchnschr., 1907, **33**, 1285.

classification. From our studies on this whole group of diseases we have come to the conclusion that there is no close relationship between the trachoma bodies and the intracellular bodies of rabies, and smallpox.

In our own work on sections, which has extended irregularly over a period of several years, we have gotten results which are somewhat confusing, principally because of the non-uniformity of the appearances of these bodies, both by different methods of demonstration and by the same methods at different times. There is no doubt that, whatever the nature of the bodies, they are easily affected by methods used for fixing, hardening, and staining them. This accounts in part for the varied results reported. However, in the most perfectly prepared specimens, judged according to the appearance of the red blood cells, leucocytes, and tissue cells at a distance from the lesions, we have found that the vaccine bodies, especially in corneal infection, show a more or less constant series of changes, somewhat similar to those described by Calkins in his "gemmul-formation" and by Tyzzer in his development of the vaccine bodies.

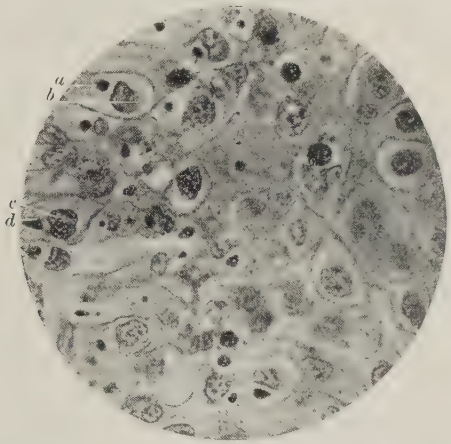


FIG. 204. — Epithelial cells of a rabbit's cornea, containing many "vaccine bodies." Tissue fixed three days after inoculation with smallpox virus. *a* and *d*, vaccine bodies; *b* and *c*, nuclei. × 1500 diameters.

Our best results on corneas have been obtained with the following technic: Fix in Zenker's fluid for from four to eight hours; wash in running water overnight; place in 95 per cent. alcohol (changing in two hours to fresh) for twenty-four hours, then in absolute alcohol for twenty-four hours. Imbed in paraffin. The cuts should be from 3μ to 5μ thick. Stain with (1) eosin and methylene blue (Mallory)—eosin half an hour, methylene blue two minutes; (2) Heidenhain's iron hematoxylin; or (3) Borrel modified by Calkins.

The vaccine bodies may be studied for a short time in the living cornea by rapidly excising an inoculated cornea, spreading it on a shallow agar plate and dropping a thin cover-glass over it. The structured bodies are very clearly differentiated from the rest of the cell contents, and interesting changes have been observed in them. Too little work has been done, however, by this method, to draw any further conclusions in regard to their nature. Councilman and Tyzzer photographed these living cornea bodies with the ultraviolet light, and the structure came out just as the chromatin structures of known living cells do.

Pathogenesis.—For Lower Animals.—Various animals seem to contract the disease, or a modification of it, in nature. Horse-pox, sheep-pox and cow-pox, all show similar pathological changes. Experimentally, probably all mammals are susceptible, though in varying degrees. Most of them are more sensitive to vaccinia than to variola. The epidermis of rabbits, for instance, shows a beautifully typical eruption after inoculation with vaccine virus, while material from smallpox eruptions produces only diffused redness. The corneal "take," however, in both instances, is similar in intensity. Monkeys are equally susceptible to both forms of the disease.

For Man.—Without vaccination human beings seem to be equally susceptible to infection with variola, whatever their race or their condition in life or in whatever part of the world they live.

Immunity.—The immunity caused by successful vaccination is not permanent, and varies in its duration in different individuals. Although it usually gives protection for several years and may give it for ten or fifteen years, it is not well to count on immunity for more than one year and whenever one is liable to exposure it is well to be vaccinated. If this vaccination is unnecessary it will not be successful, while if it is successful we have reason to believe the individual was open at least to a mild smallpox infection.

Protective Substances Present in the Serum of Animals after Successful Vaccination.—It has been frequently shown that the blood serum of a calf some days after an extensive vaccination possesses feeble protective properties, so that the injection of one or two liters of it into a susceptible calf prevents a successful vaccination. A further and more convincing fact has been demonstrated by Huddleston and others, namely, that when active vaccine is mixed in certain proportions with serum from an animal which had just recovered from a successful vaccination, and the mixture is inoculated into a susceptible animal, there is no reaction.

The Preparation of Vaccine.—The following is the method employed at the New York City Health Department.

Human-Calf-Rabbit Seed.—This is the most economic, efficient and reliable seed yet found by us. It is produced as follows: Crusts are collected from healthy children about nineteen days after successful vaccination. These crusts are cut up and emulsified with boiled water to a mucilaginous paste. This humanized seed is inoculated into an area about 6 inches square upon the abdomen of a calf, the remainder of the calf being vaccinated in the ordinary way. The pulp from this special area is separately collected and glycerinized in the usual way. It is then tested bacteriologically and clinically. This bovine virus from human seed is now used in a dilution of 1 part to $12\frac{1}{2}$ parts of normal salt solution to vaccinate rabbits. The seed is rubbed thoroughly on the freshly shaven skin of the back. Five days after vaccination the pulp is removed with a curette, weighed and emulsified in a mortar with the following solution: glycerin 50 per cent., sterile water 49.5 per cent., and carbolic acid 0.5 per cent., in the proportion of 1 part of pulp to 8 parts of the solution. Four rabbits should yield from 15 to 20 c.c. of this emulsion, an amount sufficient to vaccinate one calf.

The regular or commercial supply of vaccine is produced by vaccinating calves with this rabbit seed in the manner to be described.

Animals.—The preferable animals are female calves, from two to four months of age, in good condition, free from any skin disease and negative to the tuberculin test. These can easily be vaccinated on the posterior abdomen and insides of the thighs by placing them on an appropriate table. Older animals may be successfully employed, the only objection being that the larger animals are more difficult to handle. The virus produced is satisfactory in every way.

Vaccination.—The hair should be clipped from the entire body when the animal is first brought into the stable and the calf should be cleaned thoroughly, including the feet and the tail. Just before vaccination the posterior abdomen and insides of the thighs are shaved and the skin beneath washed in succession with soap and water, sterilized water and alcohol, and then dried with a sterile towel. On this area there are now made superficial linear incisions with a sharp knife, about a fourth of an inch apart. After they have been made they should be dried with a sterile towel or with sterile cotton and rubbed with the seed virus by means of a sterile spatula. About 20 c.c. of seed is used for each calf.

Care of the Calves.—The calves are inspected by a Department veterinarian at the time of purchase and during the seven-day period of detention previous to vaccination. After the vaccine has been collected the calves are immediately killed and their organs examined by the veterinarian. If, at autopsy, an animal be found tuberculous or otherwise diseased, the vaccine is discarded.

The vaccine stable at Otisville, N. Y., has screened windows and concrete floors and stalls. These last are kept flushed with water to wash away the dejecta of the animals. The calves stand upon raised racks of galvanized iron. They are fed on milk, no hay or straw being used for any purpose. The calves are vaccinated and the vaccine pulp collected under careful aseptic precautions in a separate operating room, which has tiled walls and a concrete floor.

Collection of Vaccine Pulp.—On the fifth or sixth day after vaccination, depending upon the rate of development of the vaccine vesicles, the calf should be ready for collection. The entire shaved area is washed with sterile water and sterile cotton and the crusts picked off. The soft pulpy mass remaining is then removed with an ordinary steel curette. The pulp is placed in a sterile bottle which is iced and sent at once to the vaccine laboratory for finishing. Brilliant green, we find, acts as a powerful germicide but has no appreciable effect on the potency of the virus. Taking advantage of this action we have applied a 1 to 1000 solution of this dye to the vaccinated area daily during the incubation period. Just prior to collection the vaccinated area is well scrubbed with soap and water, then warm water and finally sterile water. The area is then dried with a sterile towel. As a result of this treatment a marked diminution in the number of bacteria present in the pulp is noted and the final clearing in cold storage is hastened.

Preparing Vaccine Emulsion. The pulp is weighed and well mixed in a sterile mortar with four times the weight of a diluent composed of glycerin 50 per cent.; water 49 per cent.; carbolic acid 1 per cent. This mixture is forced through a 40-mesh sieve several times until no residue remains on the sieve. It is then passed through a 100-mesh sieve. The finished emulsion is stored in 100 c.c. bottles at about 5° F. At the time of grinding a 5-gram portion is emulsified with 50 per cent. glycerin in water. This emulsion is used for the test for tetanus (see below). The addition of brilliant green[1] to the glycerin-carbolic emulsion so that the final concentration is 1 to 10,000 results in a much more rapid purification of the virus. This method can be applied if haste is essential. Its drawback is the deep green color of the finished virus. The brilliant green will not kill tetanus spores. After the necessary bacteriological tests for purity (see below) the virus is filled for issue into vials or capillary tubes. The sterile vials are filled from a special burette and closed with a sterile cork. A coating of sterile paraffin is then applied as a seal to prevent evaporation and contamination. The capillary tubes sealed at one end are placed, open end down, in glass bottles in which they are sterilized. The bottles after introducing sufficient virus properly to fill the tubes, are subjected to a vacuum. By allowing the air to return slowly the virus is forced into the tubes. Through the proper adjustment of the amount of virus and the vacuum, the virus is placed in the center of the tube, leaving a clear space at each end. The tubes are then wiped dry with sterile cotton and closed by sealing the open end. All containers are carefully washed and sterilized before being used.

Testing of Finished Product.—The virus as it comes from the calf may contain the hay bacillus, skin staphylococci, possibly streptococci and perhaps B. welchii (Bacillus capsulatus aërogenes). The presence of tetanus spores is always feared but they are rarely found. After storage of the virus at the low temperatures mentioned above, the action of the carbolic, glycerine and the cold has usually freed the virus from bacteria. Before it is released for the clinical test it is subjected to the following bacteriological tests and must be free from contamination with B. welchii, B. tetani and streptococci.

Purity Tests.—(1) Plating on agar and counting of the colonies after incubation at 37° C. for two days, then reincubating at 20° C. (room temperature) and recounting the colonies.

2. Inoculation of 2 per cent. glucose broth in fermentation tubes. The presence of growth and gas are noted. Stained smears show whether or not streptococci are present, also whether any of the organisms resemble B. welchii. If so, they are tested for aërobic growth on ordinary agar. Should no growth appear the rabbit test is made.

3. Test for tetanus bacillus—Meat infusion broth in fermentation tubes is planted with a portion of the pulp ground with a water-glycerin mixture to which no carbolic has been added. The tubes are incubated

[1] Krumwiede, Fielder and Watson: Jour. Inf. Dis., 1918, **2**, 118-124.

at 37° C. for nine days. Any tube showing growth in the closed arm is used for an inoculation test in either mice or guinea-pigs. One c.c. of the nine-day culture is injected subcutaneously and the animal watched daily for six days for symptoms of tetanus.

4. *Test for Streptococcus.*—A guinea-pig is inoculated subcutaneously with the freshly prepared virus and observed for ten days for evidences of streptococcus infection.

Tests for Potency.—After all the laboratory tests for purity have been made and found satisfactory, and not before, the vaccine is ready for the test for potency. This test may be performed by diluting the virus 1 to 10, 1 to 100, and 1 to 1000 and making cutaneous insertions for each dilution on rabbits from which the hair has been removed. Tests may also be made in the same dilutions with the virus after incubation at 37° C. for twenty-four and forty-eight hours. A virus of the required potency will produce good pustules in all dilutions after forty-eight hours incubation. The potency may also be tested clinically, as is done by the Department of Health, by making ten insertions on children who have not been vaccinated before. These must all show a perfect take in order to pass the virus as up to standard. From the date of first issue until the virus is off the market clinical or animal tests should be made every two weeks. If one of these fail before the end of the period of guarantee, the vaccine is called in.

Keeping of Vaccine.—Bulk vaccine is kept in cold storage at a temperature of 4° to 8° F. The vaccine remains effective for from one to two years.

Vaccine which has been put up in packages ready for issue is kept in an ice-box at a temperature below 32° F. It should be effective for from six to twelve weeks if kept at a temperature of 41° F. or below. If left at room temperature, above 60° F., it rather rapidly deteriorates.

CHAPTER XLVI.

RABIES. YELLOW FEVER.

RABIES.

Introduction.—Rabies or hydrophobia, is an acute infectious disease of mammals, caused by a specific virus, and communicated to susceptible animals by the saliva of an infected animal coming in contact with a broken surface, usually through a bite. The name rabies (Latin) is given to the disease because of its most frequent and characteristic symptom—furor or madness, applied usually to animals. Hydrophobia (Greek, fear of water) is another name commonly used, which is also given because of a frequent symptom of the disease, the apparent fear of water, shown commonly by man. Within the gray nervous tissue of rabid animals are peculiar protozoön-like structures known as "Negri bodies" which are diagnostic of rabies. The nature of these bodies is still a question of dispute (see below).

Historical Note.—Rabies is probably one of the oldest diseases in existence, but because of the occurrence of so few human cases, and because the disease develops so long after the bite, its source was for a long time not known nor was it recognized as a separate disease. Celsus, in the first century, was the first to give in writing a detailed description of human rabies. He speaks of it being produced by the bite of rabid animals and states that the wound must be thoroughly bathed and then burned with a hot iron in order to prevent the development of the disease, for after symptoms appear death always follows.

Many hundred years passed after this without adding anything to our knowledge of the disease, though authors on the subject were numerous. Van Sweiten in 1770 observed the paralytic form of rabies in human beings. In 1802 Bosquillon emphasized the idea that belief in the existence of infectious material in rabies was a chimera and that hydrophobia was simply due to fright. This false idea had adherents for a long time; even now, by a few people, it is thought to be a true one.

Among the host of good observers who studied the disease during the latter part of the nineteenth century, Pasteur stands out as the discoverer, in 1880, of the fact that the disease may be prevented by inoculating gradually increasing doses of the virus into the person or animal bitten. This treatment with some modifications, the details of which will be given later, is still used, though many efforts have been made to develop an efficient serum treatment. Pasteur, as well as numerous other investigators, tried to discover the specific cause of rabies, but all of the results were negative.

The importance of making a quick diagnosis had become so evident that the efforts of many workers were directed toward this end alone. Pasteur and his immediate followers relied for their diagnosis entirely upon rabbit inoculations, and this meant a fifteen to twenty days' wait before the patient knew whether or not the treatment he was receiving was necessary. In 1898 this time was shortened to about nine days in our laboratory by Wilson, who found that guinea-pigs came down with the disease much more quickly than rabbits. From time to time it has been thought that certain histological

findings were diagnostic; for instance, the "rabic tubercles" of Babes, and the areas of "round- and oval-celled accumulation in the cerebrospinal and sympathetic ganglia" of Van Gehuchten and Nelis were said to be specific, but further study has shown that they are not absolutely specific for rabies. In many cases the whole picture of the grosser histological changes is sufficiently characteristic to warrant the diagnosis of rabies, but often it is not so.

It was not until Negri, in 1903, described certain bodies (Negri bodies) seen by him in large nerve cells in sections of the central nervous system, that anything was found which seemed absolutely specific for hydrophobia. Negri claims that these bodies are not only specific for rabies, but that they are probably animal parasites and the cause of the disease. We independently found the same bodies.

This work, especially so far as the diagnostic value of these bodies is concerned, has been corroborated by investigators in almost all parts of the scientific world, among them workers in our own laboratory who not only determined their worth in diagnosis, but investigated their nature.

In our work[1] emphasis was placed upon the fact that the demonstration of the "Negri bodies" by our "spread method" (see below) wonderfully simplified the process of diagnosis.

The evidence as to animal nature of these cell inclusions seemed so convincing that Williams, in 1906, gave them the name Neurocytes hydrophobiæ.[2] Calkins studied these bodies and agreed with Williams as to their nature.

A number of observers, however, still believe that the Negri body as a whole is principally the result of cell degeneration and that the specific organism may be contained within it.

Material and Methods for Study.—In New York one may still frequently obtain fresh brains of rabid animals, from veterinary hospitals or from the laboratories handling this material. Two methods have been used in helping to study the principal site of infection and to make the *diagnosis* of rabies.

(1) Animal inoculations. (2) Sections and smears.

The first method is used as a decisive test in diagnosis when results from the second method are doubtful.

The technic of the *spread method* used at present in the Research Laboratory of the New York City Health Department is as follows:

1. Glass slides and cover-glasses are washed thoroughly with soap and water, then heated in the flame to get rid of oily substances.

2. A small bit of the gray substance of brain chosen for examination is cut out with a small sharp pair of scissors and placed about 1 inch from the end of the slide, so as to leave enough room for a label. The cut in the brain should be made at right angles to its surface and a thin slice taken, avoiding the white matter as much as possible

3. A cover-slip placed over the piece of tissue is pressed upon it until it is spread out in a moderately thin layer; then the cover-slip is moved slowly and evenly over the slide to the end opposite the label. Only slight pressure should be used in making the spread, but slightly more should be exerted on the cover-glass toward the label side of the slide, thus allowing more of the nerve tissue to be carried farther down the spread and producing more well-spread nerve cells. If any thick places are left at the edge of the spread, one or two of them may be spread out toward the side of the slide with the edge of the cover-glass.

4. For diagnosis work such a spread should be made from at least three different parts of gray matter of the central nervous system: (1) From the cortex in the region of the fissure of Rolando or in the region corresponding to it in

[1] Jour. Inf. Dis., 1906, **3**, 460.

[2] Proceedings of the New York Pathological Society, 1906, **6**, 77.

lower animals (in the dog, the convolution around the crucial sulcus); (2) from Ammon's horn, and (3) from the gray matter of the cerebellum.

5. The spreads are partially dried in air and the method of fixation and staining given in Chapter III is applied to them.

With this method the Negri bodies stain magenta, their contained granules blue, the nerve cells blue, and the red blood cells yellow (Plate IX, Fig. 1).

Other methods we have found useful for staining spreads are: (1) Giemsa's (see Stains), by which, generally the "bodies" are a blue and the contained granules are azur. The cytoplasm of the nerve cells stains blue also, but with a successfully made spread the cytoplasm is so spread out that the outline and structure of most of the "bodies" are seen distinctly within it. The nuclei of the nerve cells are stained red with the azur, the nucleoli a dull blue, the red blood cells a pink yellow, more pink if the decolorization is used (Plate IX, Fig. 2). The "bodies" have an appearance of depth, due to their refractive qualities. (2) Eosin-methylene-blue method of Mallory (see Stains). With this method of staining, the cytoplasm of the Negri bodies is a magenta, light in the small bodies, darker in the larger; the central bodies and chromatoid granules are a very dark blue, the nerve-cell cytoplasm a light blue, the nucleus a darker blue, and the blood cells a brilliant eosin pink. With more decolorization in the alcohol the "bodies" are not such a deep magenta, and the difference in color between them and the red blood cells is not so marked.

In the technic of the *section* work (see Chapter III) the most important point is the time the material is allowed to remain in Zenker. According to our experience, two hours' fixation is not enough, three to eight hours is very good, and with every hour after eight hours the results become less satisfactory. Left in Zenker overnight the tissue is granular and takes the eosin stain more or less deeply, both of which results interfere with the appearance of the tiniest "bodies," especially of the very delicate, minute forms found by us in sections from fixed virus.

The sections may be stained by the eosin-methylene-blue method of Mallory (see Stains). In the sections made in this way we have been able to demonstrate clearly very minute forms, as well as good structures in the larger forms. Giemsa's method for sections may also be used. Mann's method, recommended by others, has not given us such good results.

Harris[1] published a staining method for both sections and smears, which brings the larger bodies out clearly, but which does not seem to give enough differentiation between the smaller bodies and the nucleoli of the nerve cells.

Sellers[2] published a modification of our method which seems to be an improvement, both as to definition of the bodies and quickness of technic. Sat. methyl. alc. sol. basic fuchsin, 1 c.c.; sat. methyl alc. sol. methylene blue, 15 c.c.; methyl alc. (absol. acetone-free), 25 c.c.

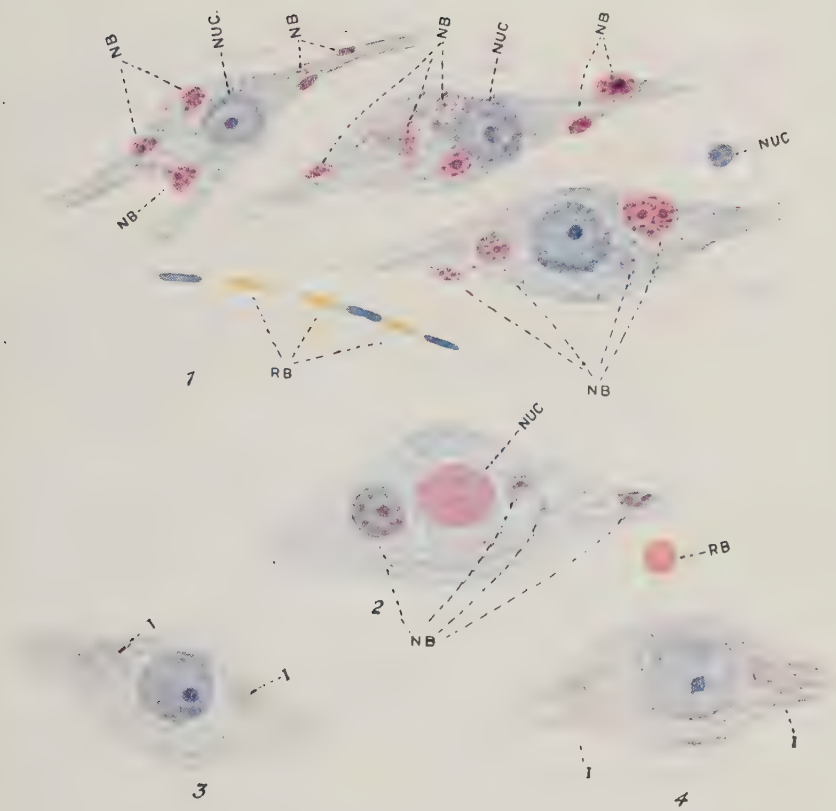
Morphology of the Negri Bodies.—The largest forms measured are about 18μ and the smallest about 0.5μ. They are round, oval, oblong, triangular, or amœboid. The latter are more numerous in the fixed virus of rabbits and guinea-pigs. Their structure is shown especially well in smears. Whatever the variety or species of animal infected, the bodies present the same general characteristic structure; *i. e.*, a hyaline-like cytoplasm with an entire margin, containing one or more chromatin bodies having a more or less complicated and regular arrangement.

Their structure varies to a certain extent with their size. In fixed virus, with an occasional exception, only tiny forms are found. These

[1] Jour. Inf. Dis., 1908, **5**, 566.

[2] Proc. of Second Annual Conf. of S. Pacif. Pub. Health Lab. Assoc., 1922, March 17, p. 33.

PLATE IX



A. W. WILLIAMS, DEL.

Nerve Cells in Spreads from Ammon's Horn.
Magnification 1200 diameters.

Figs. 1 and 2 from dog "street rabies" show "Negri bodies" (NB); Fig. 3 from non-rabic cat, and Fig. 4 from dog distemper show indefinite inclusion (I) that might be mistaken for Negri bodies by the inexperienced. Negri bodies are structured, more intensely staining, and more refractive. Figs. 1, 3, and 4 are stained by fuchsin-methylene blue mixture, which stains Negri bodies (NB) red with blue granules, nucleus (NUC) of nerve cells blue, and red-blood cells yellow (RB). Fig. 2 is stained by Giemsa's mixture, which stains Negri bodies a robin's egg blue with red granules, nucleus of nerve cells red, and red-blood cells salmon pink.

are rounded or sometimes wavy in outline, as if possessing slight amœboid motion, sometimes elongated, extending along the rim of the host-cell nucleus, or along one of the nerve fibrils, as if moving there; with eosin and methylene blue they take a delicate light magenta stain, very similar to that taken by the small serum globules in the bloodvessels. Many of the organisms, however, show a small chromatin granule, situated more or less eccentrically, sometimes on the very rim of the body. In the larger forms the granule is large, in the smaller it cannot always be seen; some of the larger forms show from two to several granules and occasionally there is a body with the definite central body and the small granules about it.

Detailed Characteristics of Structure in the Large Forms (Fig. 205).—In smears, as well as in sections, the *cytoplasm* appears quite homogeneous; there is no evidence of a reticulum or of a granular structure outside of the definite chromatoid granules. The spreads, however, have brought out one important point in regard to the cytoplasm more clearly than the sections, and that is that

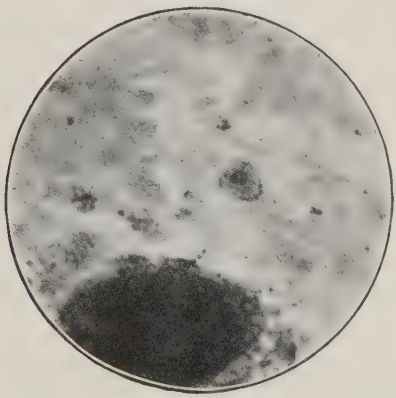


Fig. 205.—Negri body showing central chromatin with ring of small granules. × 2000.

it is more basophilic than acidophilic in staining qualities. With the Giemsa stain, as we have already seen, it takes the methylene-blue stain more than the eosin red, and even with the simple eosin-methylene-blue stain the protoplasm appears as a deep magenta unless much decolorized.

In studying the *central bodies* of these organisms, as they appear in the smears, one of the first things noticeable is that they are not surrounded by a clear space—that there is no sign of a vacuolar appearance in the body as there usually is in the sections. We notice next that in the great majority of the organisms the central body stands out clearly, as decidedly different in structure, and slightly so in staining qualities, from the chromatoid granules which surround it. The genera type of the structure of the central body is that of many well-known protozoan nuclei; that is, the chromatin is arranged in a more or less granular ring around the periphery of the central body or nucleus, leaving an achromatic or more acid-staining center in which is situated, gen rally eccentrically, a varying-sized karyosome. There are a number of variations from this principal type, according to stage of development (Plate IX, Figs. 1 and 2).

Fragmented particles seem to be leaving the nucleus in certain forms, and in this way presumably the chromatoid granules are produced.

The *chromatoid granules* are most frequently arranged in a more or less complete circle about the nucleus. They are somewhat irregular in outline and size, being occasionally ring-shaped, sometimes elongated, often in twos, due probably to active changes of growth and division. They take generally a more mixed chromatin stain than the chromatin of the nucleus.

Evidences of Division.—All stages in transverse division seem to occur. Many evidences of budding are also seen. The chromatoid granules seem to divide and pass out with part of the cytoplasm as a bud. This budding or unequal division appears to take place very early in the growth of the organism and to continue throughout growth until the parent body forms a mass of small organisms which may then break apart at the same time. The budding accounts for the number of small and large forms in a single cell (Plate IX, Fig. 1).

Number.—They vary in number according to the stage of the disease and to the infectivity of the part. They may appear in the nerve cells of the guinea-pig after "street virus" infection as early as the eighth day. After "fixed virus" infection they may be found as early as the fourth day.

Site.—They are situated chiefly in the cytoplasm and along the fibers in the branches of the large nerve cells of the central nervous system. In parts of spreads which are more broken up the bodies may appear as if lying free, and it is these bodies, if the pressure is not too great in spreading, that show the structure best. In some cases the structured bodies are distinctly localized in small scattered areas of the central nervous system. We have always found bodies in the spinal cord in abundance, but here they are especially prone to be localized in discrete groups of cells.

Manouelian[1] and Jackson[2] found them in the ganglion cells of the salivary glands.

That the organisms are present in various glands of the body (salivary, thyroid, suprarenal capsule, etc.) is shown by the virulence of emulsions from these organs. Cows' milk (Westbrook, McDaniel) and blood (Marie) have also been shown to be very occasionally virulent.

Cultivation of the Rabies Organism.—Many attempts have been made to produce artificial cultures, but no reports have been so far corroborated. Noguchi[3] stated that he obtained virulent cultures on the twenty-first transplant. Williams,[4] Gurley,[5] Krauss and Barbara,[6] and Volpino[7] have not been able to get the same results.

Diagnosis of Rabies.—(See above for procedures).—In our laboratory, for the past twenty years, or since we have used the spread method in routine diagnosis, there have been many thousand cases in all examined, including suspected rabies and controls.

In all of our work controlled by careful animal inoculations we have never yet failed to have typical rabies develop in animals inoculated

[1] Ann. de l'Inst. Past., 1914, **28**, 233.
[2] Jour. Inf. Dis., 1921, **29**, 291.
[3] Jour. Exp. Med., 1913, **18**, 314.
[4] Forscheimer's Therapeusis, 1916, 2d. ed., v, with references.
[5] Coll. Studies, New York City Health Dept., 1914, p. 15.
[6] Deut. med. Wchnschr., 1914, **40**, 1507.
[7] Presse Méd., 1914, p. 79.

with material showing definitely structured Negri bodies. Negative results after inoculation with such material must be interpreted at present as due to some error in technic, such as regurgitation, or hemorrhage at the time of inoculation, emulsion improperly made, not enough of the virulent material taken because of localization of the organisms, etc.

Possibly individual resistance of the animal inoculated might play a part. We have used principally guinea-pigs, and some of them have shown enough irregularity in regard to the time in which they have come down with the disease to suggest a varied individual susceptibility, if other factors can be ruled out.

On the other hand, material in which we have failed to demonstrate typically structured bodies has produced rabies. All of this material, however, since we have improved our technic, has shown suspicious small forms similar to those found in rabbit-fixed virus. But any decomposing brain may also show in smears, bodies very similar to these tiny forms, therefore it is difficult to rule out rabies in such cases. Of course the animal test will probably always have to be used with brains (filtered) that are too decomposed to show any formed elements except bacteria, unless a reliable chemical test can be discovered. Brains from animals dying of distemper may show small non-structured forms, somewhat like "fixed virus" forms (Plate IX, Figs. 3 and 4).

Animal's brains sent in for diagnosis should reach the laboratory as soon as possible after death. If they are a distance from the laboratory they should be packed in ice, or if that is impossible, they should be placed in glycerin. It is difficult to demonstrate the Negri bodies in decomposed brains.

So far we have not had rabies produced by fresh brains showing no Negri bodies or no suspicious forms, but a few observers have claimed that such material has produced the disease. Therefore, until we can standardize our technic, we must in all such cases use animal inoculations. We may, however, be reasonably certain that a case showing such negative material was not a case of rabies. We may summarize our knowledge in regard to the worth of the spread method in diagnosis as follows:

1. Negri bodies demonstrated, diagnosis rabies.
2. Negri bodies and suspicious bodies not demonstrated in fresh brains, not rabies.
3. Negri bodies not demonstrated in decomposing brains, uncertain.
4. Suspicious bodies in fresh brains, probably rabies.

The *localization* of the Negri bodies is an important point in making diagnosis. We have found well-developed bodies distinctly localized in different parts of the brain in several instances. In horses there may be small, widely scattered areas of well-structured forms throughout the cerebellum, while tiny, indefinite forms are scattered through the rest of the brain examined. In human brains well-developed forms may be found in the corpus striatum and not in the rest of the brain. In several dogs the localization has also been marked.

The Complement-binding Test in Rabies.—This test has been tried by Heller (1907), Friedberger (1907) and Baroni (1908) with negative results. Berry[1] (1910), and Olmstead and Wilson, in 1916, in our Research Laboratory, went over this work thoroughly and obtained similar negative results.

Effect of Chemical and Physical Agents on Rabic Virus.—Rabic virus appears to become attenuated under certain conditions of temperature; indeed, if it be subjected for about an hour to 50° C. or for half an hour to 60° C., its activity is completely destroyed. A 5 per cent. solution of carbolic acid, acting for the same period, exerts a similar effect, as do likewise 1 to 1000 solutions of bichloride of mercury, acetic acid, or potassium permanganate. Cumming corroborated the fact that formalin is especially deleterious to the virus. The virus also rapidly loses its strength by exposure to air, especially in sunlight; when, however, protected from heat, light, and air it retains its virulence for a long period.

The virus is readily filtered through all grades of Berkefeld filters, and from the glands through the coarser Chamberland. Poor and Steinhardt[2] showed that the filtered gland and brain virus seem to have similar characteristics.

Pathogenesis.—Natural Infection.—The disease occurs in nature among the following animals given in order of their frequency: dogs, cats, wolves, horses, cows, pigs, skunks, deer and man; in fact, as all warm-blooded animals are more or less susceptible to inoculations, all may presumably contract the disease when an open wound is brought in contact with infectious material of a rabid animal.

Rabies occurs in almost all parts of the world. It is most common in Russia, France, Belgium and Italy; it is not infrequent in Austria and in those parts of Germany bordering on Russia. In this hemisphere it is infrequent in Canada, but in the United States[3] cases occur with more or less frequency, according to the enforcement of laws regarding the control of dogs. In Mexico and South America it occurs occasionally; while in England, North Germany, Switzerland, Holland and Denmark, because of the enforced quarantine laws, and of the wise provision that all dogs shall be muzzled, it is extremely rare. In Australia it is unknown, probably because the law that every dog imported into the island must first undergo a six months' quarantine has always been enforced. After 1915 it decreased markedly in New York City for a time, due to the fact that the muzzling ordinance was more enforced. In 1926 it showed a marked increase.

In this connection the question as to how long the sputum of a rabid dog may remain virulent after it drops from the animal is an interesting one. A case came under our observation in 1906 which illustrates this point. A child of six years came down with typical rabies in a neighborhood where there had recently been several cases of canine rabies, but no history of a bite could be

[1] Jour. Exp. Med., 1910, **12**, 338. [2] Jour. Inf. Dis., 1913, **13**, 203.
[3] Kerr and Stimson: Jour. Am. Med. Assn., 1909, **53**, 989; Rice and Beatty, Am. Jour. Pub. Health, 1928, **18**, 421.

obtained. The parents were sure she had not been bitten. Six weeks before, however, the child had fallen in the street and cut her cheek severely on a jagged stone. The wound was cauterized and healed without further trouble. A mad dog had been on that street just before this occurred. It is reasonable to suppose that the stone had on it some of the sputum from that dog, and so the child was infected. Such a case would not occur very often, but the possibility should be considered.

In regard to the question as to whether the bite of apparently healthy animals may give the disease, it may be said that, judging from laboratory experiments, some animals may have a light attack of the disease and recover spontaneously; though such cases, if they occur, are probably extremely rare. That the bite of an infected animal may give the disease before that animal shows symptoms has been proved. Fifteen days is the longest time reported between the biting of a human being and the appearance of symptoms in the dog. Therefore, if an animal is kept under observation three weeks after biting another, without developing symptoms, he may be pronounced free from suspicion of having infected sputum. One weeks' observation would be sufficient for the vast majority of cases.

Neither age, sex, nor occupation has any specific effect. The time of the year seems to have little effect, though most cases are said to occur during the summer months. The numbers vary with different years.

The certainty with which the disease may be produced after a bite and the rapidity of its development have been found to be governed by certain factors: (1) the point of inoculation; (2) the quantity of the rabic virus introduced; (3) the strength of the virus and the susceptibility of the animal bitten. It is a matter of common observation that in man slight wounds of the skin of the limbs and of the back or wherever the skin is thick and the nerves few either produce no results, especially when bites are made through clothes, or are followed by the disease after an extremely long period of incubation; while in lacerated wounds of the tip of the fingers where small nerves are numerous or where the muscles and nerve trunks are reached, or in lacerated wounds of the face where there is also an abundance of nerves the period of incubation is usually much shorter and the disease generally more virulent.

These facts explain why only about 16 per cent. of human beings bitten by rabid animals and untreated appear to contract hydrophobia.

Cases have been reported from time to time of spontaneous recovery from developed rabies,[1] but the diagnosis in such cases is difficult to make; so we are still in doubt as to this taking place. It should, however, be borne in mind.

Since the establishment of the Pasteur treatment for the disease, the percentage of developed cases after bites is very much less—a fraction of 1 per cent.

Symptoms.—There is always a decided incubation period after the bite which varies within quite wide limits, but in the majority of cases it is from twenty to sixty days. Any period after six months is an exception; the shortest we have on record is fourteen days and the longest authentic period is seven months.

[1] Philip, Barry and Snook: Jour. Inf. Dis., 1921, **29**, 97.

A very few apparently authentic cases have been reported as developing in about one year, but reports of any time beyond this must be received with doubt.

The symptoms may be divided into three stages: (1) The prodromal or melancholic stage; (2) the excited or convulsive stage; and (3) the paralytic stage.

When the second stage is the most pronounced the disease is called furious or convulsive rabies; when this stage is very short or practically lacking and paralysis begins early, the disease is called dumb or paralytic rabies.

In the Dog.—The principal symptoms of each form may be summarized as follows: (*a*) *Furious rabies:* change of behavior, biting (especially at those to whom the animal has been affectionate before), increased aggressiveness, characteristic restlessness, loss of appetite for ordinary food, with desire to eat unusual things, intermittent disturbance of consciousness, paroxysms of fury, peculiar howling bark, rapid emaciation, paralysis, beginning in the hindlimbs, death in great majority of cases in three to six days (exceptionally slightly longer) after the beginning of symptoms. (*b*) *Paralytic rabies:* short period of excitation, paralysis of the lower jaw, hoarse bark, appetite and consciousness disturbed, weakness, with paralysis spreading in great majority of cases, and death four or five days after first symptoms. There may be a number of cases showing transition types between these two forms.

In Human Beings.—*Furious Rabies.*—The first definite symptoms are difficult and gasping breath with a feeling of oppression and difficulty in swallowing, the latter the most characteristic symptom. It is caused by convulsive contraction of the throat muscles. The attacks are brought out when attempting to drink or swallow. The very thought of drinking may bring one on; and though there is no fear of water itself, there is fear of taking it because of the effect it produces. The convulsive attacks finally become more or less general over the whole body; in certain cases some parts are more affected by reflex excitation than others; for instance, there may be slight or no photophobia, while in exceptional cases, more frequently in dogs, the hydrophobia is also absent.

Most of the special reflexes are increased. Pupils become irregularly contracted and widened until they finally remain fixed.

The temperature is increased from 38° to 40° C., at first with morning remissions. Just before death it may rise as high as 42.8° C. The pulse is generally over 100 and is irregular. This stage lasts from one to four days. Death may occur during a convulsion, but more often there is a *paralytic stage*, which lasts from two to eighteen hours. The convulsions become less frequent and the patient becomes weaker until finally there is a complete paralysis. At the beginning of this stage the patient may be able to drink water better than formerly. Death may occur at any time through paralysis of the heart or respiratory center.

Paralytic Rabies.—This form occurs quite seldom in human beings, more frequently in dogs, but not so often as the convulsive form. It is supposed to occur in humans and dogs after a more severe infection. Instead of periods of convulsions, the various muscles simply tremble and become gradually weaker until complete general paralysis supervenes. Sometimes paralysis develops very quickly and may be general before death from syncope or asphyxia occurs. This form generally lasts longer than ordinary rabies. Between these two typical forms of rabies there are many different types, giving quite varied pictures of the disease.

Length of the Disease.—The majority of the cases of furious rabies die on the third or fourth day after the symptoms show themselves. The limits of the reported cases are one to fifteen days, though there are reports of only one or two cases dying on any day after the ninth to the fifteenth. As the time when the symptoms really begin is difficult to notice, these statistics are probably only approximately correct. In paralytic rabies the average time in which death occurs is five days.

Treatment.—All wounds should be immediately cleaned and when possible should be thoroughly cauterized with *fuming nitric acid.* Even forty-eight hours after infection this may help. In the case of small wounds all the treatment probably necessary will be thorough cauterization with nitric acid within twelve hours from the time of infection. Our experience in dealing with those bitten by rabid animals goes to show that physicians do not appreciate the value of thorough cauterization of the infected wounds.

Pasteur's Method of Preventive Inoculation.—Pasteur's treatment is based upon the fact that rabic virus may be attenuated or intensified under certain conditions. He first observed that the tissues and fluids taken from rabid animals varied considerably in their virulence. Then he showed that the virus may be intensified by successive passage through certain animals (rabbits, guinea-pigs, cats) and weakened in passing through others (monkeys). If successive inoculations be made into rabbits with virus, either from the dog or the monkey, the virulence may be so exalted beyond that of the virus taken from a street dog, in which the incubation period is from twelve to fourteen days, that at the end of the fiftieth passage the incubation period may be reduced to about six or seven days when it remains fixed. This "*fixed virus*" was used by Pasteur and those after him in his preventive treatment because the dose could be more definitely regulated by subsequent attenuation or dilution.

Present Administration of Pasteur's Treatment in Human Beings.—Pasteur's method in its entirety was soon adopted in many lands, and his results were corroborated. Before long, however, a number of modifications were suggested by different observers, some slight, others more fundamental. Some have been widely used, such as Högyes' dilution method;[1] others have had a limited application in lower animals and are probably only of theoretic interest as regards man. Such are the intravenous inoculation of brain emulsions from street rabies into herbivora (Nocard and Roux, Protopopoff), and the intraperitoneal inoculations of large doses of fully virulent fixed virus into dogs, cats, or rabbits (Hellman, Heim, Remlinger). Immunity has been produced also in rats by allowing them to feed on rabid brains (Fermi, Repetto, Remlinger.) (See also Marie[2] for bibliography).

Högyes in Budapest was one of the first to use a different procedure. He claimed that the virus by Pasteur's method was attenuated only through the death of some of the specific organisms, that is, that there were simply fewer living organisms in the early doses given than in the later, and that therefore the same result might be obtained perhaps with even more accurate dosage by giving gradually decreasing dilutions of a fresh virulent cord. By diluting sufficiently he obtained a mixture which when inoculated did not produce rabies in the test animals, a result similar to that following an eight- to ten-day dried cord. This dilution he used for the first inoculation and gradually stronger dilutions for the succeeding ones.

Philips[3] recommended a dilution method of using a glycerinated vaccine which we tested and found effective.

Other methods of attenuating or diluting fixed virus have been used, such as exposure to the action of heat, cold, gastric juice, glycerin, carbolic acid or

[1] Nothnagel's Specielle Path. u. Ther., Wien, 1897.
[2] L'Etude expérimentale da la Rage, Paris, 1909.
[3] Jour. Immunol., 1922, 7, 409.

ether. The ether method[1] has been tested out recently in India[2] with marked success.

Technic of Pasteur's Method.—The cord is removed by a modification of the method of Oshida in the following manner: Strict asepsis is preserved. The rabbit when completely paralyzed (seventh day) is killed by gas or chloroform and is dropped into a 5 per cent. solution of carbolic acid for five minutes. It is then removed, the excess of carbolic solution is drained off, and an incision through the skin at the upper and inner part of the thigh is made. The skin is loosened by cutting around the lower portion of the trunk. It is then pulled by the hands toward the upper extremity of the animal and over the head to the ears, leaving the back exposed and sterile throughout the entire length of the spine. The spine is then divided transversely near each extremity by bone-cutting forceps. The muscles are cut through about these areas so the spine may be more easily reached. With a long wire probe swabbed with cotton at one end the cord is pushed upward from its canal, freed from its nerves and membranes. The spine is steadied by lion-jawed forceps. The cord curls in a spiral as it emerges and rests on the sterile muscles of the neck. It is lifted with forceps, placed in a Petri dish and cut in two. A small piece is cut from one end and is dropped into a tube of broth to test its purity. A ligature with one long end is placed about each piece, both of which are then hung in a drying bottle (Fig. 206).

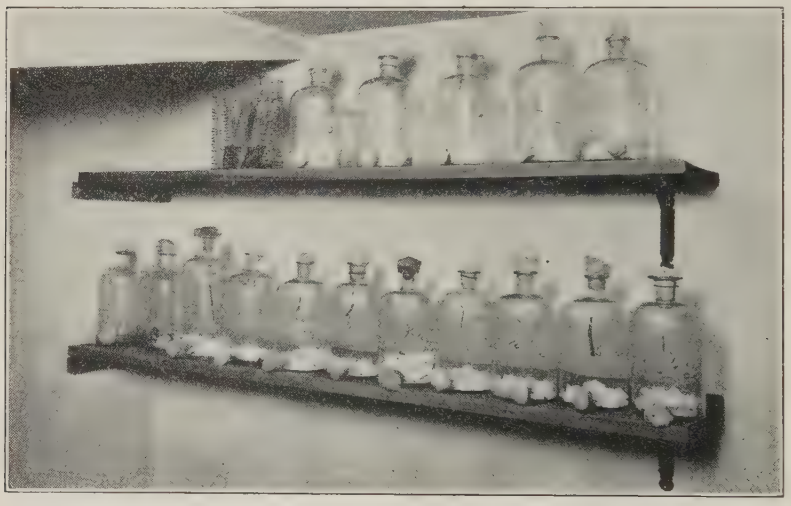


Fig. 206.—One corner of constant temperature room showing drying bottles containing fixed virus cords being prepared for vaccine.

Drying the Cord.—The drying bottles are sterile aspiration bottles with both openings plugged with cotton. A layer 1 inch high of sticks of caustic potash covers the bottom, and the pieces of cord are suspended from the top cotton plug by their attached ligatures. The bottles are then labelled and placed in the constant temperature room (Fig. 206) or incubator, which is kept at a temperature of about 22° C. (70° F.). After twenty-four hours' drying the cord is known as one-day cord; after two days, two-day cord, etc. Pieces of cord cut off at any time and put into glycerin will retain about the same strength for

[1] Alirisatas: Dent. med. Wchnschr., 1922, **48**, 9295.
[2] Cunningham, Nicholas and Larkin: Ind. Jour. Med. Res., 1928, **16**, 245, 253, 259.

several weeks. This procedure is followed in regions where there are few cases of rabies, and the daily killing of rabbits to keep up the vaccine would be a large expense. It may also be followed where treatment is sent by mail.

The New York City Health Department used Pasteur's first schedule, with modifications, up to January, 1906, when they began treatment with a ten- and nine-day cord and finished with a one-day. They continued with this until August, 1913 when they began using the more intensive method of the Hygienic Laboratory at Washington. From 1906 to 1921 inclusive they treated 6738 cases infected by rabid animals, with a total mortality of 0.47 per cent. and a corrected mortality of 0.17 per cent. They had 7 cases of definite paralysis with 2 deaths; 6850 cases in all, including those not bitten by rabid animals, were treated.

Since it had been found that fresh rabbit-fixed virus inoculated subcutaneously into man is apparently harmless, the Berlin Institute, with the hope of obtaining an earlier immunization and a shorter treatment, began to give still earlier cords. In 1901 it began with the eight-day cord on the first inoculation, and was inoculating a two-day cord on the eighth day of treatment. Its treatment lasted twenty-one days. This method was adopted at the Hygienic Laboratory in Washington in 1908, with slight variations for the different degrees of bites.

In 1919 Semple[1] reported good results from the use of a vaccine made with dilutions of the virus killed with phenol (Fermi) and slight heat.

Tyler in our laboratory tested the efficiency of this vaccine and found it gave remarkably good results. This vaccine contains less nerve tissue than the old vaccine and its dosage is more accurate. It is more conveniently made and distributed. For those reasons our Health Department changed to this method of treatment on August 23, 1926.

The vaccine consists of a sterile 4 per cent. emulsion of killed rabies fixed virus from the brains of rabbits. The virus is killed by incubating an 8 per cent. emulsion of the brain virus in 1 per cent. phenol normal salt solution (0.8 per cent.) for twenty-four hours at 37° C. The emulsion is then diluted with an equal volume of the saline and bottled in 2 c.c. vials. Tests are made in susceptible animals to prove the absence of virulent virus. The course consists of a daily injection of 2 c.c. for fourteen days.

The injections are made subcutaneously usually over the abdomen.

Treatment by Mail.—The New York City Health Department was the first to send out treatment by mail to physicians for their own patients. Full directions are sent with the mailing case.

The results from the treatment sent in this way seem to be equally as good as those from the treatment administered at the laboratory.

Rapid Drying of Rabies Virus.—Harris, of St. Louis, published a new method of drying rabies virus and of regulating the dosage.

Technic.—The brain and cord are removed aseptically and ground up in a sterile mortar with a sufficient quantity of CO_2 snow thoroughly to freeze the tissue. The frozen nerve tissue and snow are then placed in a Scheibler jar over H_2SO_4, the jar being kept in a Frigo apparatus. A vacuum of from 5 to 2 mm. is produced in the jar, which is then kept at the temperature of 18° C. by an ice and salt mixture for a sufficient length of time to dry thoroughly the nerve substance, which then appears as a dry powder. About two days are required for one brain and cord, which lose about one-half of their virulence in the process. The powder is then sealed in tubes *in vacuo* and kept at a temperature below 0° C. until required for use. It has been found that by keeping the powder thoroughly dry and cold practically no further loss of virulence

[1] Brit. Med. Jour., 1919, ii, 333 and 371.

occurs for at least six months. Before storing the virus for use its strength in units is computed, the unit being the minimal infecting dose (M. I. D.) for a rabbit when injected intracerebrally.

The advantages claimed for this method are: (1) the ease and economy with which a large amount of virus can be prepared, it being necessary to prepare the virus for use even in large laboratories only at intervals of several months; (2) the possibility of more accurate dosage for the patients; (3) a shortened period of treatment; and (4) the inoculation of more virus units. The required amount of powdered virus is weighed out each morning, and the necessary dilutions in salt solution for the various patients are made from this.

Fixed Virus Modified by Dialysis.—Cumming, of Ann Arbor, has devised a method of antirabic vaccination, by which he uses fixed virus which has been rendered avirulent by dialysis.

The emulsion of fixed virus is placed in collodion sacs (prepared by the Novy method and sterilized in the autoclave at 105° C. for twenty minutes) and dialyzed in distilled water for from twelve to twenty-four hours. The resulting vaccine does not produce rabies on intracranial inoculations, but does produce immunity on subcutaneous inoculations. Experiments by Cumming on rabbits show that whereas the original Pasteur method protects against only twice the minimum lethal dose (minute directions for obtaining the M. L. D. are given) injected intracerebrally, and the Högyes method against one and one-half times the fatal dose, the dialysis method protects against at least three times the fatal dose. He also claims that immunity is produced at an earlier date than by the other methods. Treatment (2 c.c. of the vaccine) is given daily for from fifteen to twenty-five days. Cumming reports over 800 cases (62 per cent. bitten by animals proved to have been rabid) treated without a death and without complications.

Marie's Method.—For several years the use of virus serum mixture was in vogue at the Pasteur Institute in Paris, the technic of which is as follows:

1 gm of the medulla of a rabbit dead of fixed virus is finely emulsified with 9 c.c. of 0.8 per cent. salt solution and filtered through linen. Two c.c. of this emulsion and 4 c.c. of antirabic serum (obtained from sheep, and inactivated at 56° C. for thirty minutes) are carefully mixed after standing for a time. Six c.c. of this mixture, which contains an excess of virus is injected into the patient. These injections are repeated on the next three days, after which the treatment proceeds according to the regular Pasteur schema, beginning with the use of a six-day cord on the fifth day. The antirabic serum is obtained from sheep which have been subjected to a long and strong course of treatment with fixed virus. It is claimed that a quicker immunity is produced by the serum-virus mixture than by the original Pasteur schema, an advantage of especial value in the treatment of cases liable to become infected with a short incubation, such as bites on the head.

Antirabic Serum.—The possibility that the serum of animals immunized against rabies contains protective substances was suggested by Pasteur as early as 1889. The following year Babes recommended the use of the serum of vaccinated animals in combination with the Pasteur treatment. Since then the study of the amount and character of the antibody content of animals immunized against rabies has been carried

on more or less extensively both from the theoretic and the practical sides. It was hoped that a serum could be obtained that would effect a cure for developed rabies just as diphtheria antitoxin does for developed diphtheria. But such a definite applicability of the serum has not developed. It was soon found that, while serum of certain vaccinated animals possessed the property of neutralizing rabies virus *in vitro*, it had only a slight inhibiting power when inoculated into the living animal, and apparently no action at all by any method of inoculation after the disease had become manifest. Babes still claims, however, that the serum has enough effect *in vivo* to be used in treatment, and his serum treatment is based upon this claim. He gives as his reason for employing serum at the end of treatment that he wishes to introduce into the patient at the time he most needs it the largest amount of antibodies. He also claims that the serum so given will prevent or cure the occasional paralyses which occur during treatment.

Those who did not agree with Babes were led to test the practical use of the serum combined with the beginning vaccine inoculations.

Remlinger, Marie and others showed that a serum-virus mixture with a slight excess of virus will protect an animal against infection into the anterior chamber of the eye when inoculated during the three days following the vaccination. Thus Marie showed that immunity is produced more quickly by these unsaturated mixtures of virus and serum than by the virus alone. If a surplus of serum is present the animals are not protected from a later infection.

Marie, who has used the serum in his treatment of humans since 1904, prepares it as follows:

> The brains of two rabbits dying from fixed virus infection are finely rubbed up with physiologic salt solution in the proportion of 20 gm. in 180 c.c. This emulsion is filtered through fine cloth and heated for one-half hour at 37° C. Sheep are used for the inoculation. Each sheep receives intravenously 30 c.c. (3 gm. fixed virus) a week for six to eight weeks. Thirteen days after the last inoculation the first blood is drawn. Then in a period of two weeks, at 4 bleedings, 200 c.c. of blood are drawn. After a fourteen-day pause another series of inoculations are given and the animal is ready for another series of bleedings. From each animal yearly about 3 liters of antirabic serum are obtained. A strong serum is one that neutralizes 40 virus units in 1 c.c.

A *virus unit* is 1 c.c. of five times the dilution of fixed virus that will surely kill a rabbit inoculated intracerebrally, *e. g.*, the unit of a fixed virus that will surely kill a rabbit in 1 to 500 dilution is 1 c.c. of a 1 to 100 dilution.

The nature of the antibodies in rabies serum has been the subject of many studies. Fermi and a few others claim that the antibodies are not specific. They say that they can obtain a similar serum after the inoculation of normal brain emulsions. Some even use normal brain emulsions in the treatment of their lighter cases.

Certain investigators (Kraus, Marie and others), while not able to corroborate all of these claims, have found that the sera of certain animals which are more or less refractory to rabies possess a small amount of rabicidal strength; *e. g.*, 0.5 c.c. of normal chicken serum

mixed with one unit of fixed virus (1 c.c. of 1 to 100 dilution) causes the latter to become neutral in eighteen hours.

All species of animals tried produce the specific antibodies, but not to an equal degree. Human beings and monkeys are said to have more antibodies after vaccination than rabbits.

Centanni showed that immediately after vaccination the animal is not fully protected, though its serum may contain antirabic qualities, while later the animal is immune, though its serum may not be able to neutralize the rabies virus. These facts point to a cellular immunity.

Results of Antirabic Treatment.—On the whole the results of protective inoculations against rabies are marked. One has only to compare the statistics of mortality after bites from animals suffering from hydrophobia with those given after any of the methods of treatment employed to see the benefit. As regards the best method to use, the case is different. With many methods tried in many lands on a large number of cases, it would seem that we should be able by this time to determine their comparative worth. But the trouble is that the improvement on the whole is not great and the statistics are not kept uniformly or minutely enough to draw trustworthy comparisons.

A slight decrease in mortality has been shown in the statistics from most of the antirabic institutes of the world.

But these figures tell us little about the actual value of the different methods. In order to be able better to judge, the statistics should uniformly give many more details. Some institutes give such details, others do not. Until some such scheme as the following is carried out by all, we must change cautiously a treatment that has already given good results.

1. Diagnosis of biting animal:
   (*a*) Rabies, (*b*) probably rabies, (*c*) questionable, (*d*) not rabies, (*e*) nothing known.
2. Manner of making diagnosis:
   (*a*) By animal inoculation, (*b*) by microscopic examination, (*c*) by clinical diagnosis.
3. Site and character of bites (*e. g.*, number, depth, laceration, protected by clothing, etc.):
   (*a*) Head, (*b*) hands, (*c*) other parts of body.
4. Time elapsing between bite and beginning of treatment.
5. Method of treatment used.
6. Complications during or after treatment, particularly paralysis.
7. Character and time of death.

That the time after the bite makes a great difference is shown by the following table:

	Time intervening between bite and beginning treatment	Number of cases treated.	Death.	Percentages.
Babes	1 to 2 days	3406	3	0.088
	3 to 5 days	2541	2	0.077
	5 to 6 days	809	1	0.124
Diatroptoff	1 week	4602	26	0.560
	2 weeks	961	16	1.660
	3 weeks	313	10	3.190

IMMUNITY.—The immunity in human beings produced by the antirabic treatment apparently lasts a variable time. That it may not last more than fourteen months is shown by the history of one of our cases. The patient was an assistant in a hospital for dogs. He was given eighteen days' treatment after a light wound in the hand from a rabid dog. Fourteen months later he came down with typical hydrophobia. Since his treatment he had become very careless with cases of rabies, exposing wounded hands to saliva because he considered himself immune. He was warned that there might be danger. Six weeks before his death he put a wounded hand into the mouth of a rabid animal. There is little doubt that this is a case of reinfection after loss of protection from the treatment rather than one of delayed hydrophobia.

Marie has found complete immunity in dogs eighteen months after treatment.

Ill Effects of Treatment.—LOCAL.—There is only slight local discomfort, increased a little if the emulsion contains glycerin. During the second week an erythema often appears about the point of inoculation, which Stimson regards as a manifestation of hypersusceptibility to foreign nerve tissue. It disappears in a few days.

CONSTITUTIONAL.—Ever since the beginning of treatment occasional non-fatal affections of the nervous system have been reported, which occurred during or shortly after the course of treatment. These have varied in degree all the way from a slight neuritis, through paraplegias to paralyses of various parts of the body. Very occasionally the paralyses are marked and the patient dies. Cases of true paralytic rabies which may occur within the period required for the establishment of immunity by the treatment must be differentiated from cases occurring as a result of treatment.

Simon published an extensive report of 84 cases of paralysis occurring during the years 1888 to 1911 inclusive, and Fielder[1] gave a corroborative report in 1916.

In analyzing the effect of different methods of treatment on paralyses, Simon gives the following summary:

	Number of cases treated.	Cases of paralysis.	Proportion.
Classic Pasteur method	32,676	6	1 in 5446
Modified Pasteur method	8,657	16	1 in 541
Högyes method	51,417	3	1 in 17139

It is seen that the number of paralyses following the Höyges method are markedly less than those following the other methods.

We have had no cases of paralysis following our treatment since 1916.

From the studies so far made of these paralyses the possibility of there being different causes for different cases cannot yet be ruled out. The chief theories advanced as to factors in producing the condition are six:

1. Due to "laboratory rabies" from the fixed virus vaccine inoculated.

[1] Jour. Am. Med. Assn., 1916, vol. 66.

2. Due to "modified rabies" resulting from the treatment on the street virus infection.

3. Due to a toxin produced by the rabies organisms.

4. Due to infection with extraneous organisms introduced with the virus during treatment.

5. Due to psychological disorders.

6. Due to the inoculation of a foreign protein.

Preventive Measures in Animals.—Far more important than any treatment, curative or preventive, for hydrophobia in man is the prevention of rabies in dogs, through which this disease is usually conveyed. Were all dogs under legislative control and the compulsory wearing of muzzles rigidly enforced for two years where rabies prevails, hydrophobia would practically be stamped out. This fact has been amply demonstrated by the statistics of rabies in countries (*e. g.*, England) where such laws are in force. New York City from time to time has had some measure of success in enforcing such laws.

Umeno and Doi[1] recommended giving each licensed dog one or at the most two large doses of a carbolized vaccine. The results while encouraging[2] are not always satisfactory[3], since it fails to protect at times and the duration of the immunity is usually less than a year.

YELLOW FEVER.

Yellow fever is an acute infectious disease of tropical countries, with no characteristic lesions except jaundice and hemorrhage. Other lesions that exist are those common to toxemia. One attack usually produces complete immunity.

Historical Note.—There have been many extensive studies on the etiology of this disease with numerous announcements of the discovery of its specific cause. Not one of the latter, however, has been corroborated.

The epoch-making investigations of the United States Army Commission composed of Walter Reed, James Carroll, Aristides Agramonte and Jesse W. Lazear[4] (1901) established the truth, that this disease, like malaria, is carried from one infected person to another through the agency of a mosquito. Nott, in 1870, and Finley, in 1881, each asserted that the mosquito was the transmitter of the disease. They were, however, unable to prove their theory, and it remained for the commission conclusively to show that a distinct species of mosquito carried the infection.

The work of the American commission was fully corroborated by the French commission and by other workers.

The principal facts established by the commission were summed up by Goldberger[5] as follows:

1. Yellow fever is transmitted, under natural conditions, only by the bite of a mosquito (*Aedes egypti*) that at least twelve days before

[1] Kitasato's, Arch. Exp. Med., 1921, **4**, 89.
[2] Eichhorn: Am. Jour. Pub. Health, 1926, **16**, 644.
[3] Hermann: Centralbl. Bact. Abt 3, 1928, **107**, 84.
[4] Jour. Exp. Med., 1900, **5**, 215; Jour. Am. Med. Assn., 1901, **36**, 413.
[5] The Yellow Fever Institute Bulletin No. 16 with review of literature to 1907.

has fed on the blood of a person sick with this disease during the first three days of his illness.

2. Yellow fever can be produced in man under artificial conditions by the subcutaneous injection of blood taken from the general circulation of a person sick with this disease during the first three days of his illness.

3. Yellow fever is not conveyed by fomites.

4. *Bacillus icteroides* Sanarelli stands in no causative relation to yellow fever.

Certain facts in regard to the nature of the specific parasite were brought out by these studies:

1. It seems to require two hosts (a mammal and an arthropod) for the completion of its life cycle (analogies, *Plasmodium malariæ*, *Babesia bigeminum*).

2. There is a definite time after the bite of the mosquito before the blood of the person bitten becomes infective (average five days), and a definite time that the blood remains infective (three days).

3. The blood during these three days is still infective after passing through the finest-grained porcelain filters (Chamberland B and F).

4. The blood loses its virulence quickly (forty-eight hours) when exposed to the air at a temperature of 24° to 30° C. When protected from the air by oil and kept at the same temperature it remains virulent longer (five to eight days). Heated for five minutes at 55° C. it becomes non-virulent.

5. The bite of an infected mosquito does not become infectious until twelve days (at a temperature of 31° C.) after it has bitten the first patient.

The cause of the disease is still under discussion. Noguchi's[1] spirochete is now considered only a secondary invader. In the infective blood filtrates have been found only small dancing granules with the dark-field illumination similar to those found in healthy persons, with the exception that Simpson reported the finding of a spirochete.

Some of the species of the higher monkeys are susceptible.

Cowdry[2] has just reported the finding of intranuclear inclusions in yellow fever monkeys, similar to those found by Taires[3] and by Stokes, Bauer and Haddon.[4]

The Yellow-fever Mosquito (Figs. 207 to 212).—The name *Stegomyia* for this small tropical mosquito was suggested by the English entomologist Theobald, who separated this genus from the genus *Culex*, with which it was formerly classed. It was first given the specific name *Fasciata*, but Blanchard proved that this had already been used and the name *Calopus* (Meigen, 1818) was thought to be the proper one. Later the genus *Stegomyia* was shown to be invalid and the organism then went by the name *Aëdes calopus* Meigen. Calopus was then shown to be invalid, so now the name is *Aëdes egypti*. Salient characteristics of

[1] Jour. Exp. Med., 1919, **29**, 565 and **30**, 1, 9 and 13.
[2] Science, 1929, **69**, 252.
[3] C. R. Soc. Biol., 1928, **99**, 1344, 1655, 1660, 1664, 1671.
[4] Am. Jour. Trop. Med., 1928, **8**, 103.

Aëdes are: (1) The palpi in the male are as long or nearly as long as the proboscis; in the female the palpi are uniformly less than one-half as long; (2) the legs are destitute of erect scales and are alternately banded white and black; (3) the thorax is marked with lines of silvery scales. *Aëdes egypti* is spread over a wide range of territory, embracing many varieties of climate and natural conditions. It has been found as far north as Charleston, S. C., and as far south as Rio de la Plata. There is no reason to believe that it may not be present at some time or other in any of the intermediate countries. In the United States specimens of *Aëdes egypti* have been captured in Georgia, Louis-

FIG. 207.—The yellow-fever mosquito (*Aëdes egypti*). Adult female. Much enlarged. (Howard.[1])

iana, South Carolina and eastern Texas. The island of Cuba is overrun with this insect. The fact that *Aëdes egypti* has been known to exist at various times in Spain and other European countries may account for the spread of yellow fever which has occurred there once or twice in former times; the same may be said of the country farther north in the United States, where *Aëdes egypti* has not yet been reported, but which have suffered from invasions of yellow fever.

[1] Farmers' Bulletin, No. 547, U. S. Dept. of Agric., Washington, D. C.

FIG. 208.—The yellow-fever mosquito. Adult male. Much enlarged. (Howard.)

FIG. 209.—The yellow-fever mosquito. Adult female, side view. Much enlarged. (Howard.)

FIG. 210.—The yellow-fever mosquito. Egg. Greatly enlarged. (Howard.)

The adult mosquito is so small that mosquito netting of 19 or 20 strands or meshes to the inch is required to keep the insect from entering a place.

Brackish water is unsuited for the development of *Aedes* larvæ. The species *Aëdes egypti* seems to select any deposit of water which is comparatively clean. The defective drains along the eaves of tile roofs are a favorite breeding place in Havana and its suburbs; indoors they find an excellent medium in the water of cups of tin or china into which the legs of tables are usually thrust to protect the contents

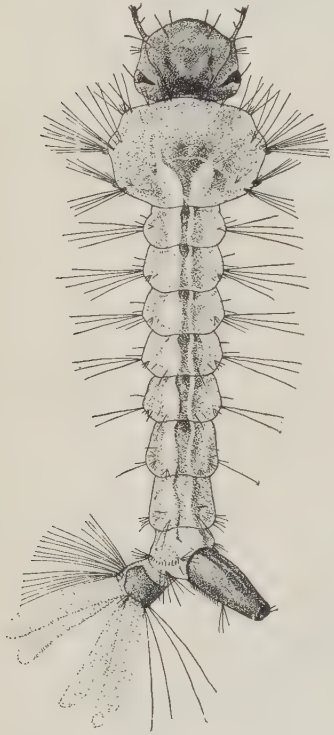


Fig. 211.—The yellow-fever mosquito. Larva. Much enlarged. (Howard.)

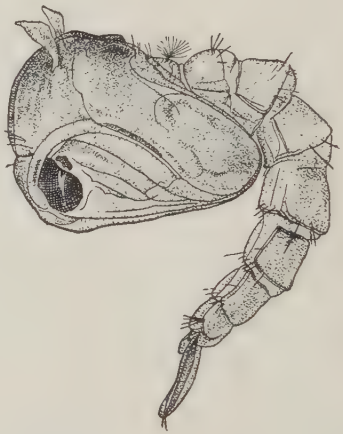


Fig. 212.—The yellow-fever mosquito. Pupa. Much enlarged. (Howard.)

from the invasion of ants, a veritable pest in tropical countries. The same may be said of shallow traps, where the water is not frequently disturbed.

Like other *Culicidæ*, it prefers to lay at night. It is eminently a town insect, seldom breeding far outside of the city limits. Agramonte never found *Aëdes egypti* resting under bushes, in open fields, or in the woods; this fact explains the well-founded opinion that yellow fever is a domiciliary infection.

The question of hibernation in the larval stage is important. Agramonte failed to get larvæ that could resist freezing temperature, and

found that in the case of *Aëdes egypti* this degree of cold was invariably fatal.

The possibility of their being capable of life outside their natural element must also be considered from an epidemiological point of view. The dry season in the countries where this species seems to abound is never so prolonged as completely to dry up the usual breeding places. Experimentally, adult larvæ removed from the water and placed overnight upon moist filter paper could not be revived the following morning.

The question of the life period of the female insect is of the greatest importance when we come to consider the apparently long interval which at times has occurred between the stamping out of an epidemic of yellow fever and its new outbreak without introduction of new cases. The fact is that *Aëdes egypti* is a long-lived insect; one individual was kept by Agramonte in a jar through March and April into May, in all for seventy-six days after hatching in the laboratory.

These mosquitoes bite principally in the late afternoon, though they may be incited to take blood at any hour of the day. They are abundant from March to September, and even in November Agramonte was able to capture them at will in his office and laboratory.

The mosquito is generally believed to be incapable of long flight unless very materially assisted by the wind. At any rate, the close study of the spread of infection of yellow fever shows that the tendency is for it to remain restricted within very limited areas, and that whenever it has travelled far beyond this, the means afforded (railway cars, vessels, etc.) have been other than the natural flight of the insect.

Experiments have demonstrated that not all mosquitoes which bite a yellow-fever patient become infected, but that of several which bite at the same time some may fail either to get the parasite or to allow its later development in their body. This condition is similar to that seen in *Anopheles*, with regard to malaria.

The question as to the length of time infected mosquitoes remain dangerous to the non-immune community cannot be definitely answered at present; there is good presumptive evidence that the mosquito may harbor the parasite through the winter and be enabled to transmit in the spring an infection acquired in the fall. There is reason to believe that the mosquito, once infected, can transmit the disease at any time during the remainder of its life. Freezing temperature, however, quickly kills the insect.

Carrying out preventive measures based on the knowledge gained by the splendid work of the American Army Commission, yellow fever has been practically wiped out of Cuba, the Isthmian Canal Zone and many other infected areas.

PART III.

APPLIED MICROBIOLOGY.

CHAPTER XLVII.

THE BACTERIOLOGICAL EXAMINATION OF WATER, AIR, AND SOIL. THE CONTAMINATION AND PURIFICATION OF WATER. THE DISPOSAL OF SEWAGE.

EXAMINATION OF WATER.

The bacteriological examination of water, while it is undertaken chiefly for the purpose of discovering whether any pathogenic bacteria for man are liable to be present, rarely actually seeks them, but rather other bacteria which suggest pollution from fecal discharges and, therefore, the possibility of dangerous contamination. The determination of the number of bacteria in water was for a time considered of great importance, then it fell into disrepute, and the attempt was made to isolate the specific germs of diseases which were thought to be water-borne. At first these attempts seemed very successful in that supposed typhoid bacilli and cholera spirilla were found. Further study revealed the fact that there were common water and intestinal bacteria which were so closely allied to the above forms that the tests applied did not separate them. When proper identification was carried out the very great majority of the suspected organisms were found to be non-pathogenic. The improbability of getting pathogenic bacteria from suspected water except under unusually favorable conditions caused a return to the estimation of the number of bacteria in water and above all to the estimation of the number of intestinal bacteria. It is known that the coli aërogenes group of bacilli have a somewhat longer existence than the typhoid bacilli, and as the colon bacilli appear in large numbers in the intestinal passages of men and animals, it was fair to assume that typhoid bacilli, dysentery or other pathogenic bacteria could not occur from fecal pollution without the presence of the colon bacillus except in rare cases.

The usual microbes in water can be divided into those which live in water and those which, while commonly inhabiting the soil, are frequently transported to the water. There are a great number of varieties of these. They are much more abundant in surface waters than in spring or well waters. The presence of some of these microbes

causes an unpleasant taste or smell, but otherwise they are harmless. In inhabited regions water is subject to pollution from discharges from sick persons and carriers and from animals. We fear those from persons especially. In this climate we fear the typhoid and paratyphoid bacilli especially, in other climates the dysentery bacilli, the cholera spirilla and protozoa.

The attention of sanitarians has been devoted to the interpretation of the presence of smaller or larger numbers of colon bacilli in water, until, at present, the examination of water is based upon the quantitative analysis (measuring, within certain limits, decomposing organic matter) and the colon test (indicating more specifically pollution derived from intestinal discharges of man or animals).

Technique for Bacteriological Examination.—The utmost care is necessary in order to get reliable results. A speck of dust, a contaminated Petri dish, a considerable delay, improperly sterilized agar or gelatin, a temperature either too high or too low, any of these factors may introduce an error or variation in results which would make a reliable test impossible.

Collection of Samples.—The small sample taken must represent the whole from which it is drawn. If a brook water, it must be taken some distance from the bank; if from a tap, the water in the pipes must first be run off, for otherwise the effect of metallic substances will invalidate the results; if from lake or pond, the surface scum or bottom mud must be avoided, but may be examined separately. The utensils by which the water is taken should be of good quality glass, clean and sterile. From a brook the water can be taken directly into the bottle, the stopper being removed while it fills, but avoiding the surface film and its attending excessive numbers of bacteria; from a river or pond it can be taken from the bow of a small boat, or from a bottle properly fastened on the end of a pole so as to avoid contamination; from a well a special apparatus has been devised by Abbott, a bottle with a leaded bottom is so held that when lowered to the proper depth a jerk will remove the cork and allow the bottle to fill. The same device or another accomplishing the same purpose can be rigged up readily by anyone. The sample of water should be tested as soon as possible, for some of the bacteria immediately begin to increase while others decrease. In small bottles removed from the light, predatory microörganisms and many bacteria begin to increase, and among these are the members of the colon group. Thus, the Franklands record a case in which a sample of well water kept during three days at a moderate temperature showed a bacterial increase from 7 to 495,000, while Jordan reported a sample in which the bacteria decreased in twenty-four hours from 535,000 to 54,500. In a sample we kept at room temperature the colon bacilli during twenty-four hours increased from 10 to 100 per c.c. The only safe way to prevent this increase is to plate and plant the water in fermentation tubes within a space of one or two hours or to keep it at a temperature under 5° C. (41° F.). If it cannot be kept cold, it is far better to make the cultures in the open field or in a house

rather than to wait six to twelve hours for the conveniences and advantages of the laboratory.

The third matter of great importance is the addition of proper amounts of water to the broth in the fermentation tubes and to the media in the plates. Usually five fermentation tubes are inoculated as follows, 10 c.c., 5 c.c., 1 c.c., 0.1 c.c., and 0.05 c.c., while the plates are inoculated with 1 c.c. and 0.1 c.c. each and poured with 10 c.c. of standard nutrient agar. If possible, duplicate tests should always be made. When it is desired to know whether colon bacilli are present in larger amounts than 10 c.c., quantities up to 100 c.c. can be added to bouillon, and then after a few hours' incubation, 1 c.c. added to fermentation tubes. Less than 20 colonies and more than 200 on a plate give inaccurate counts, the smaller number being too few on which to base an average and the larger number interfering with each other. When as many as 10,000 colonies develop in the agar contained in one plate, it will be found that there will develop in a second plate containing but one-tenth the amount of water from 20 to 50 per cent. as many colonies. This shows that the crowding of the colonies had prevented the growth or caused a fusion of one-half to four-fifths of them.

The chemical composition of the medium on which the bacteria are grown affects the result of the analysis. Nutrient 1.5 per cent. agar gives slightly lower counts than gelatin, but on account of its convenience in summer and its greater uniformity it is being used more and more generally for routine quantitative work.[1] A uniform standard is a necessity if comparable results are to be secured. (See Media for Water.) At best only a certain proportion of bacteria develop, and it is important only that our counts represent a section through the true bacterial flora of quick-growing sewage forms. Comparability is the vitally essential factor. Counting is done with a lens of $2\frac{1}{2}$ diameters' magnification $3\frac{1}{2} \times$ with a focal length of $3\frac{1}{2}$ inches.[2]

The temperature at which the bacteria develop is of great importance; also they should be protected from light. The access of oxygen which prevents the growth of anaërobes must not be forgotten. As a rule, the plate cultures are developed at two temperatures. The Standard Methods calls for two days at 20° to 21° C., and for twenty-four hours at 37° C. Some bacteria do not develop colonies in two days, but these are neglected. The number of bacteria growing at room temperature is usually much greater than those growing at 37° C. As all the groups of intestinal bacteria grow at body temperature, while many of the water types do not, some investigators believe it important to develop the bacteria at both temperatures so as to compare the results. We have not found this to be of any advantage in detecting intestinal types when the more specific tests are made for the colon group of bacilli.

The lactose broth is incubated at 37° C. for detection of members of

[1] Standard Methods of Water Analysis. American Public Health Association. Sixth edition. Third impression, 1928. American Public Health Association.

[2] The engraver's lens No. 146, Bausch & Lomb Optical Co., fills these requirements.

the coli-aërogenes group. The fermentation tubes not showing gas after forty-eight hours' incubation are recorded as negative and discarded. If no gas is formed in twenty-four hours or the amount formed is less than 10 per cent of the content of the closed arm the tubes should be incubated another twenty-four hours. The presence of gas in any amount in such tubes constitutes a doubtful test and requires confirmation. The formation within twenty-four hours of gas occupying more than 10 per cent of the tube constitutes a *positive presumptive test.* Confirmation is carried out as follows: Make an endo- or an eosin-methylene blue agar plate and streak from fermentation tube showing gas in smallest portion of each sample. Incubate at 37° C. Organisms of the coli-aërogenes group ferment lactose and thus produce acid, which is shown by the indicator in the medium. At least two colonies showing the acid reaction are fished and planted into lactose bouillon and onto agar slants. The coli-aërogenes group as here defined consists of organisms of both fecal and non-fecal origin, and care must be exercised in judging the sanitary quality of water solely from this data. Unfortunately there are some types of bacilli growing in the soil which resemble them. If it is necessary to obtain a more accurate presumption the cultures should be subjected to the Voges reaction (page 148), the methyl red test or the sodium citrate test.[1] An acid reaction with the methyl red test, and negative Voges reaction and failure to grow on the sodium citrate medium indicates that a strain is of fecal origin. A complete correlation in the results with the above tests is not to be expected. For a more complete understanding of the technique and the interpretation of results of the bacteriological examination of water see *Elements of Water Bacteriology,* by Prescott and Winslow and the Standard Methods of the American Public Health Association.

The presence of spore-bearing gas-forming bacilli, especially B. welchii may result in spurious positive presumptive tests. Lactose bile peptone medium has been suggested to prevent this. This medium has been largely abandoned because of the inhibition of the growth of coli-aërogenes types. Hale advises the use of a medium containing 5 per cent of bile and 1 to 10,000 brilliant green. He believes that the inhibition of B. welchii in this medium is of more sanitary importance than the inhibition of some attenuated coli-aërogenes types, and that the attenuated types have little sanitary significance.

Significance of the Presence of Coli-aërogenes Group.—The test for the presence of these types has been received by the majority of engineers and practical sanitarians with great satisfaction, and has been applied with confidence to the examination not only of water, but of shell-fish and other articles of food as well. In some instances apparent contamination may be shown by bacteriological examination in waters where inspection will reveal no sources of fecal pollution. In such instances the coli-aërogenes types found on completer tests will give the reactions of the non-fecal varieties. Many animals other than man

[1] Koser: Jour. Bacteriol., 1924, **9**, 59.

have the coli-aërogenes group in their intestinal tract, such as horses, cows, swine, fowl, etc. The washings from manured soil is a common source of contamination of water. This contamination cannot be differentiated by laboratory test from the potentially dangerous fecal pollution from human sources. Inspection alone can indicate the probable sources, and, in general, waters should not be condemned on a bacteriological examination alone. Inspection may also indicate that water is not contaminated at its source. Thus, a well may be contaminated by surface wash and when this is prevented the water will be of high sanitary quality. In general, significance should be given to the numbers of coli-aërogenes bacilli rather than their presence in any body of surface water. The absence of coli-aërogenes bacilli in water proves its harmlessness so far as bacteriology can prove it. When coli-aërogenes bacilli are present, so as to be isolated from 1 c.c. of water in a series of tests, it is reasonable proof of animal or human pollution and the conditions should be investigated. Ten coli-aërogenes bacilli in 1 c.c. indicates serious pollution. Surface waters from inhabited regions will always contain numerous colon bacilli after a heavy rainstorm or shower. Well and spring water should be free of coli-aërogenes bacilli.

The experience of all who have studied the subject practically is that, in delicacy, the coli-aërogenes test surpasses chemical analysis; in constancy and definiteness it also excels the quantitative bacterial count.

Interpretation of the Quantitative Bacteriological Analysis.—The older experimenters attempted to establish arbitrary standards by which the sanitary quality of water could be fixed by the number of germs alone. Sternberg considered that a water containing less than 100 bacteria is presumably from a deep source and uncontaminated by surface drainage; that one with 500 bacteria is open to suspicion; and that one with over 1000 bacteria is presumably contaminated by sewage or surface drainage.

The tendency today is to emphasize the coli-aërogenes test and to give less consideration to the colony count, except in the control of water purification. It is not included in the standards recommended by the Advisory Committee of the United States Public Health Service.[1] The source of the sample is of importance in any attempt at interpretation; thus, a bacterial count which suggests that a spring or well is contaminated might be normal for a river. In woodland springs and lakes several hundred bacteria per cubic centimeter are frequently found. In lakes the point at which the sample is taken is of great importance, as the bacterial count varies with the distance from the shore and with the depth. The weather also is an influence, since the wind causes currents and waves which stir up the bottom mud, bringing up organisms which have been sedimented. Rains greatly influence streams by flooding them with surface water, bringing a huge number of bacteria at times. The season of the year is an important factor. The counts are highest in the winter and spring months and lower from April to September.

[1] Public Health Reports, 1925, **40**, 693.

The following figures illustrate this point:

Water.	Observer.	Year,	Jan.	Feb.	Mar.	April.	May.	June.
New York City tap water .	Houghton	1904	890	1100	650	240	350	370
New York City	Noble	1916	13	17	50	33	6	13
Boston tap water . . .	Whipple	1892	135	211	102	52	53	86
Merrimac River tap water .	Clark	1899	4900	5900	6300	2900	1900	3500

The winter and spring increases are due to melting of the snow or rains on frozen ground resulting in a heavy flow of surface water into the streams without the usual filtering soil percolation. These seasonal increases are direct indications of possible danger. A number of severe epidemics of typhoid fever have been produced in this way. It is only the fact that typhoid fever is at a minimum in winter that prevents more frequent typhoid pollution. Although, as a rule, a series of tests are necessary to pass judgment on a water, a single test may be very important. A large increase in the number in tap water a day after a storm points unerringly to surface pollution, and if towns exist in the watershed, to street and sewer pollution. The untreated Croton water frequently jumps from hundreds to thousands after such a storm. The low bacterial content in 1916 is due to the addition of chlorine to the water after leaving the reservoir.

In a typhoid epidemic at Newport, Winslow found that the water supply showed but 334 bacteria per cubic centimeter, but one from a well showed 6100. The suspicion aroused was justified by finding that all the typhoid cases had obtained water from this well.

It is evident from the above that the total count may have considerable indicative value. However, where the total count rises due to the contamination from fecal sources, the coli-aërogenes test will show a similar rise.

The study of the bacterial content of water before and after filtration is the only way in which the efficiency of the filter can be determined and the cause of accidents ascertained. In Germany these regular tests are obligatory. The filter should remove about 99 per cent. of the bacteria. Elaborate studies have been made of the exact distribution of streams of sewage in bodies of water into which they flow, their disappearance by dilution and sedimentation, and their removal by death. Under peculiar conditions bacteria in water may increase for a time, but here the prevailing bacteria belong almost exclusively to one type.

Bacteria Other Than of the Coli-aërogenes Group.—Most of the bacteria which develop in the intestines of man and animals necessarily occur in polluted water, and an examination for some of these has been advocated by many, such as the Clostridium welchii, other anaërobic spore formers, the various members of the typhoid-colon, proteus, pyocyaneus and streptococcus groups. Houston recommended the examination for streptococci and for B. welchii. Both these varieties, especially the latter, will persist in water after coli-aërogenes types have disappeared. These examinations are made seldom today. The examination for B. welchii may be indicated where acute enteritis is apparently due to contaminated water.

Isolation of the Typhoid Bacillus from Water.—If it were possible to obtain readily the typhoid bacilli from water, when they are present in small numbers, such an examination would be of great value. The difficulties are that we can examine at one time only a relatively small amount of water by bacteriological methods, and under ordinary conditions the numbers of *B. typhosus* are small, but the numbers of associated bacteria will be proportionately very high. Likewise, by the time cases develop and are reported the bacilli may have mostly disappeared from the water, unless contamination is continued.

In the light of present-day experience the most hopeful procedure would be to concentrate the bacilli in the water and inoculate brilliant green broth containing graded amounts of brilliant green and to inoculate plates of Krumwiede brilliant green agar from the broth daily for five to seven days. (See Chapter XXI, *B. typhosus*.) Concentration of the bacilli may be done by centrifugalizing at very high speed or by filtering through small Berkefeld filters, the candles being then placed in the broth. Suspected cultures, if obtained, must be studied in complete detail, including agglutinin absorption to eliminate any possible error due to group agglutination. This possibility is not excluded in many of the reported isolations of *B. typhosus*, and the identity of the cultures isolated is, therefore, in doubt.

CONTAMINATION AND PURIFICATION OF DRINKING WATERS.

Brook water and river water are contaminated in two ways: by chemicals, the waste products of manufacturing establishments, and by harmful bacteria from the contents of drains, sewers, etc. The latter method is by far the more dangerous.

When water, which has been soiled by waste products of manufactories only, becomes so diluted or purified that the contamination is not noticeable to the senses and shows no dangerous products on chemical analysis, it is probably safe to drink. When sewage is the contamination this rule no longer holds, there may be no chemical impurities and no pathogenic bacteria found and yet disease may be produced. That river water which has been fouled by sewage will, by oxidation, dilution, sedimentation, action of sunlight, and predatory microörganisms, become greatly purified is an indisputable fact. Where contaminated river water is drawn into storage reservoirs the above factors, except dilution, will lead to further self-purification. The increase in bacteria which occurs from contamination is also largely or entirely lost after ten to twenty miles of river flow. Nevertheless, the history of many epidemics seems to show that a badly contaminated river is never an absolutely safe water to drink, although with the lapse of each day it becomes less and less dangerous, nor will sand filter-beds absolutely remove all danger. These statements are founded upon the results of numerous investigations. The marked disappearance of bacteria is illustrated by the following: Kummel found below the town of Rosbock 48,000 bacteria to the cubic centimeter; twenty-five kilo-

meters farther down the stream only 200 were present—about the same number as before the sewage of Rosbock entered. On the other hand, the doubtful security of depending on river purification is proved by such experiences as the following: In the city of Lowell, Massachusetts, an alarming epidemic followed the pollution of the Merrimac River three miles above by typhoid feces, and six weeks later an alarming epidemic attacked Lawrence, nine miles below Lowell. It is estimated that the water took ten days to pass from Lowell to Lawrence and through the reservoirs. Typhoid bacilli usually die in river water in from three to ten days, but they may live for twenty-five days, so the Lawrence epidemic is easily explained. Newark-on-Trent, England, averaged 75 cases a year from moderately well-filtered water and only 10 when it changed to deep-well supply.

Purification of Water on a Large Scale.—For detailed information reference should be made to works on hygiene. In general, three procedures are available for the purification of surface waters: storage, filtration and chlorination. The selection of the procedures to be employed is determined by local conditions, space and availability of materials. Storage in reservoirs, due to the factors already described, will result in self-purification and the degree will be dependent on the storage time. If the time is long enough the added procedure of chlorination will give a completely safe water. Where storage can only be of short duration or is not feasible at all, filtration is resorted to. Two types of filtration are used, slow sand filtration and mechanical sand filtration. In slow sand filtration large areas of sand (about 3 feet deep) are used. On the surface a slimy layer, rich in oxidizing microorganisms, is formed, and mechanical filtration and oxidation combine to render the process 98 to 99.8 per cent efficient. If the available area is small, mechanical filtration is done through smaller areas of sand under pressure, a coagulant of organic matter, sulphate of aluminum, being added to the water before filtration. This procedure is the more adaptable to waters requiring clarification. A slow sand filter will deliver daily from 1,000,000 to 2,000,000 gallons of water, a mechanical filter will deliver 100,000,000 gallons or more. The effluent from filters is not a completely safe water and chlorination should be done to render it so. Chlorination is carried out by the addition of from 1 to 10 parts of available chlorine to one million of water either in the form of chlorine gas or chlorinated lime. The amount required depends on the total organic matter content in the water.

Domestic Purification.—Water which requires private filtering should not be supplied for drinking purposes. Unhappily, however, it often is. Domestic filters may be divided, roughly, into those for high and low pressure. The former are connected directly with the water main, while the others simply have the slight pressure of the column of water standing in the filter. Many household filters contain animal charcoal, silicated carbon, etc., either in a pressed condition or in one porous mass. These filters remove much of the deleterious matter from the suspected waters, but the majority cannot be depended upon to remove

all bacteria. Even those which are equipped for self-cleansing become foul in a little while, and, if not cleaned, unfit for use. The best of the filters are of porous stone, such as the Doulton-Berkefeld and Pasteur filters. These yield a water, if too great pressure is not used, almost absolutely free from bacteria, and if they are frequently cleansed they are useful. A large Berkefeld filter will allow 60 gallons of water to pass per hour. The Pasteur filter is more compact and slower. From the best Pasteur filters sterile water may be passed for two or three weeks; from the Berkefeld usually for a few days only. Whenever water is suspected, and there is any doubt as to the filters, it should be boiled for ten minutes; this will destroy all bacteria.

The Importance of Ice in the Production of Typhoid Fever.—The total number of instances of typhoid fever which have been reasonably attributed to ice infection are remarkably few. Such an instance was a small epidemic which occurred among those who used ice from a pond. It was found that water directly infected with typhoid feces had flowed over its frozen surface and been congealed there.

Life of the Typhoid Bacillus in Ice in Laboratory Experiments.—The first important investigation was that of Prudden, who showed that typhoid bacilli might live for three months or longer in ice. This experiment is frequently wrongly interpreted, as when a writer states: "It has been amply demonstrated that the germs of typhoid fever are not killed by freezing and that they have been known to live in ice for long periods of time."

It is true that in Prudden's experiment a few typhoid bacilli remained alive for three months, when the experiment was terminated, but those were but a small fraction of 1 per cent. of the original number. Following Prudden's experiment Sedgwick and Winslow, in Boston, and Park, in New York City, carried on independently a series of experiments. These led to the same conclusions. A table summarizing a final experiment of ours in which twenty-one different strains, mostly of recent isolation, were subjected to the test is given below:

LIFE OF TWENTY-ONE STRAINS OF TYPHOID BACILLI IN ICE.

	Average number of bacilli in 1 gm. of ice.	Percentage typhoid bacilli living.
Before freezing	2,560,410	100.0
Frozen three days	1,089,470	42.0
Frozen seven days	361,136	14.0
Frozen fourteen days	203,300	8.0
Frozen twenty-one days	10,280	0.4
Frozen five weeks	2,950	0.1
Frozen nine weeks	127	0.005
Frozen sixteen weeks	107	0.004
Frozen twenty-two weeks	0	0.0

In these experiments twenty-one different flasks of Croton water were inoculated each with a different strain of typhoid bacilli. In one a little of the feces rich in typhoid was directly added. The infected water in each flask was then pipetted into thirty tubes. These tubes

were placed in a cold-storage room in which the temperature varied from 20° to 28° F. At first tubes were removed and tested twice a week, later once a week. The reason for using so many different strains was because it has become evident that some cultures live longer than others.

At the end of five weeks the water infected with six cultures was sterile, at the end of sixteen weeks only four strains remained alive.

Interesting investigations of Hudson River ice were carried out in 1907 by North.

There was noticed a considerable difference between the number of bacteria in the top, middle, and bottom layers of ice. This is natural, since, while water in freezing from above downward markedly purifies itself, 75 per cent. of the solids and a fair proportion of bacteria being eliminated, yet this cannot happen in the case of the snow blanket which becomes flooded by rain or by cutting holes through the ice. Here all impurities, such as dust and leaves which have fallen on the surface and dirt which may come from the water, remain with the bacteria which they carry, since all are retained in the porous snow. The bacteria in freshly cut bottom ice generally show the least destruction by freezing.

The river water in the forty specimens averaged 1800 bacteria per cubic centimeter, the top ice 306, the bottom ice 36 and the middle ice 14. Only four specimens of top ice had over 500 bacteria per cubic centimeter; none of the specimens of middle or bottom ice had that many.

The great destruction by freezing is noticeable in these figures. Even the top ice soiled by flooding and by the horses and men gathering it contained but 16 per cent. as many bacteria as the water from which it was obtained. The bottom ice, the last to be frozen, had but 2 per cent. of those in the water.

Conclusions in Regard to Ice Pollution.—The danger from the use of ice produced from polluted water is always much less than the use of the water itself. Every week that the ice is stored the danger becomes less, so that at four weeks it has become as much purified from typhoid bacilli as if subjected to sand filtration; at the end of four months the danger becomes almost negligible and at the end of six months quite so.

THE DISPOSAL OF SEWAGE.

The disposal of sewage is becoming a vital question with all towns and cities, since the present tendency in legislation is to compel such towns to dispose of their waste in such manner that it shall not contaminate drinking water, shell fish and bathing beaches, and that it shall not be destructive to fisheries, or a nuisance to harbors.

Methods of sewage purification depend upon the character of the sewage and the kind of effluent desired. For details reference should be made to books on hygiene.

Briefly outlined, screening, sedimentation or precipitation, digestion and oxidation, filtration and final chlorination may be employed. After removal of refuse the sewage is tanked. Various types are employed to obtain sedimentation and putrefactive activity on solid organic matter to give an effluent for further treatment. The Imhoff tank is a well-known form. The effluent of the tank may then be treated on sprinkling filters. This process depends upon saturation of the sewage with oxygen by spraying it in the air and then allowing it to trickle over stone beds, about 6 to 10 feet deep, made up of broken stone, $1\frac{1}{2}$ to $2\frac{1}{2}$ inches in diameter. Fine screening prior to filtration is being used as a substitute for tanks. If the final effluent must be treated further to obtain complete clarification sand filtration is employed; settled sewage or the effluent of sprinkling filters is run onto the sand bed. Chlorine should be added if the effluent must be discharged into waters used for drinking purposes, using 10 to 20 parts of available chlorine per 1,000,000 gallons of clarified sewage. Where an effluent is discharged into waters which will not be used for drinking purposes, nonputrescibility of the effluent only is aimed at.

Sea Water.—This is only feebly bactericidal. In salt water 99 per cent of typhoid bacilli will die in five days. A very few may survive for twelve to nineteen days.

BACTERIA IN AIR—EXAMINATION OF AIR.

Saprophytic bacteria are always present in considerable numbers in the air except far out at sea and on high mountains. They reach the air from the earth's surface and are most plentiful nearest to it. They are more abundant where organic matter abounds and in dry and windy weather.

The air is kept constantly in motion by winds so that fine particles are being carried into it continually from the ground, especially in an inhabited area with its dusty streets. The rays of sunlight visibly reveal these particles to us. The bacteria in the dust of the fields and streets are carried along with these dust particles. They are usually the harmless soil bacteria or the almost equally harmless intestinal bacteria of animals. After a rain few bacteria are in the air, while on a dry windy day many thousands exist in a cubic meter. The number of bacteria in the air of the country is much less than in the city air. Forests decrease the number of bacteria.

On high mountains and on the sea far from land, bacteria are very scarce. The bacteria that multiply in the soil of street and country are almost entirely saprophytic types. Sunlight and drying rapidly destroy bacteria. In dwellings the bacterial content depends on many factors, of which the chief are the opening of windows to the outside dust-laden air, the cleanliness of the dwelling, and the amount of stirring up of the dust by sweeping. It is almost impossible to separate the effect of the bacteria which we inhale from that of the dust particles

which they accompany. Both probably act as slight irritants and so predispose to definite infections.

Except when in direct contact with men and animals, the air contains pathogenic bacteria only under exceptional circumstances and usually as spores, such as those of anthrax bacilli in dust from the wool and hides of infected animals or of tetanus bacilli from infected manure. The practical results obtained from the examination of air for pathogenic bacteria have been slight. We know that at times they must be in the air, but unless we purposely increase their numbers they are so few in the comparatively small amount of air which it is practicable to examine that we rarely find them. It is established that in loud speaking, in coughing and sneezing many bacteria from the larynx, fauces and lips are expelled. Examination of dust in hospital wards and sick rooms, in places where only air infection was possible, has occasionally revealed tubercle bacilli and other pathogenic bacteria. Although very light they generally settle to the ground. It is now thought that the factor of air infection in the spread of infectious diseases is of very little importance, except when recent droplet infection has occurred. In the tiny droplets of mucus most of these bacteria die quickly and do not disseminate, while alive, far from the place of their origin.

The simplest method of searching for the varieties of bacteria in the air and their number in any place is to expose to the air for longer or shorter periods poured plates of nutrient agar. After exposure the plates are either put in the incubator at 37° C., or kept at room temperature. When it is desired to obtain parasitic bacteria blood agar may be used. The more careful quantitative examination is made by drawing a given quantity of air through tubes containing clean sterile sand, which is kept in place by pieces of metal gauze. When the operation is completed the sand is poured into a tube containing a measured quantity of water and after thorough shaking aliquot parts of the water are quickly removed and plated with nutrient agar, each poured into a Petri dish and the bacteria allowed to develop, either at 37° or 23° C., according as the growth of the parasitic or saprophytic varieties is desired. Instead of nutrient agar or gelatin, blood agar, ascitic broth or animals may be inoculated. Such examinations are occasionally made of the air of theatres, crowded streets in cities, etc. They give usually the number of non-pathogenic bacteria only.

BACTERIOLOGICAL EXAMINATION OF THE SOIL.

The subject from its agricultural side is considered in Chapter L. Specimens of deep soil can be gathered in sterile, sharp-pointed, sheet-iron tubes. Through the examination we wish to learn either the number of bacteria or the important varieties of bacteria present. To estimate the number, small fractions of a gram are taken and planted in nutrient agar or in special media contained in Petri dishes. Anaërobic as well as aërobic cultures should be made.

According to Houston, uncultivated sand soil averages 100,000 bacteria per gram, garden soil 1,500,000, and sewage-polluted 115,000,000. The most important bacteria to be sought for are bacilli of the colon group and streptococci. Both of these suggest fairly recent excremental pollution. Tetanus spores may also be found.

The period during which typhoid bacilli remain alive in soil is variable, since it depends on so many unknown factors and differs so in different places. The typhoid bacilli probably rarely increase in the soil and probably rarely survive a month in it. Pathogenic bacteria in the soil are dangerous mainly because they may be washed into water supplies by rains or carried to them by the wind.

CHAPTER XLVIII.

THE BACTERIOLOGY OF MILK IN ITS RELATION TO DISEASE.

From the standpoint of the dairy many of the different varieties of bacteria found in milk are of importance which have little or no medical interest. We have space here only to consider the bacteriology of milk so far as it is related to health and disease.[1] The parasitic bacteria of the cow taken collectively have importance because one can determine from their number something as to the care taken in handling the milk at the time of milking, and they, together with the saprophytic bacteria, when numerous, produce chemical changes in the milk, some of which are harmful to infants. The bacilli of the colon group derived from healthy cattle are of little more importance than many of the saprophytic types. However, they indicate pollution from the cow which is potentially harmful.

Numerical Estimation of Bacteria.—The number of bacteria in a cubic centimeter of milk is usually estimated from the colonies developing in standard nutrient agar plate cultures at 37° C. during a period of forty-eight hours. This allows only a certain proportion of the varieties of bacteria to develop colonies, as many of the bacteria in market milk prefer a lower temperature. Sometimes fully twice as many colonies develop at 20° to 27° C. during three to five days as at 37° C. for two days. The advantages of the shorter time and of uniformity have led to the adoption of the technique given in Part I. Any culture method necessarily underestimates their number, as many of the bacteria remain, after vigorous shaking, in pairs or small groups. In order to overcome this and also to note the morphological types, the direct microscopic examination of smears of the sediment has been urged. A great practical objection to this is that, if a heated milk is examined, the dead as well as the living bacteria are counted, for no satisfactory staining method has yet been found to differentiate between those already dead and those killed at the time of fixing the film to the slide and in staining. This method has, however, great advantages at the creamery or farm in that one can tell immediately whether a sample has few or many bacteria and also note the presence of streptococci and leukocytes. When the individual bacteria are counted the microscopic count gives from two to ten times as many bacteria as the routine culture method. When clumps of bacteria are given the same value as single bacteria the microscopic counts agree rather closely with the colony counts. The important thing in the estimation of the number of bacteria in milk is to

[1] Fermented milks are discussed in the chapter on the Microörganisms of the Alimentary Canal.

use standard methods[1] so that uniform results will be obtained by all careful technicians. The agar plate count is in general use and is recognized as standard by the American Public Health Association. The direct microscopic count has now been admitted by the above Association as a standard procedure for suitable milks.

Smear Method for Direct Examination of Milk.—The Prescott-Breed[2] method is usually employed. Recently Newman made a decided improvement in the staining method. (See below.)

Advantages and Disadvantages of the Direct or Microscopic Method of Milk Analysis.—Advantages.—1. The counting of each organism seen, while difficult and time-consuming, makes it possible to ascertain approximately the actual number of bacteria in milk and to determine the proportions of the various types. The counting of the clump as one organism approximates the colony count, but loses the value of an exact estimation.

2. It eliminates the greater portion of glassware required at present in the plate method, the preparation of agar, etc., which lessens the initial cost.

3. It gives a Department of Health a ready means of control of the milk at its source in the country creameries and aids the field inspectors in the sanitary control of the dairies.

These advantages, however, accrue mainly to the dealer employing a bacteriologist to make the milk analyses at the source.

Disadvantages.—The disadvantages of the direct method are: 1. The very small quantity of milk usually used for counting leads to inaccuracy.

2. That as a resultant of the foregoing fact large factors have to be used in estimating the bacterial content of milk, with the consequent introduction of large factors of error.

3. In estimating at all accurately the bacterial content of a very good milk, rather large numbers of fields have to be examined with a consequent expenditure of time, which increases the cost.

4. The individuality of the counter enters more largely into this method than into the plate method, and a better trained technician is necessary. The counts given in the table represent fairly the difference in the counts in the two methods.

Samples graded on colony count, bacterial content per c.c.	No. of samples.	Average plate count.	Average microscopic count of individual organisms.	Ratio.
Less than 50,000 . . .	594	14,491	92,034	1:6.29
50,000 to 300,000 . .	133	103,075	708,681	1:6.87
300,000 to 1,500,000 . .	42	648,452	5,254,018	1:8.1
1,500,000 to 5,000,000 . .	13	2,538,461	21,541,534	1:8.48
5,000,000 or over . . .	8	6,575,000	27,519,531	1:4.18
Total	790	171,181	1,100,997	1:6.43

[1] Standard Methods for the Bacteriological Examination of Milk, American Public Health Association.

[2] Centralbl. f. Bakteriol., Parasiten u. Infectionskrankheiten, 1911, Abt. II, Nos. 16, 18, **30**, 337.

Prescott and Breed describe their smear method briefly as follows: The sample of milk to be examined is shaken thoroughly and 0.01 c.c. is withdrawn by means of a specially constructed capillary pipette. The milk so obtained is spread evenly over an area of one square centimeter on an ordinary glass slide. These areas may be easily determined by placing the glass slide over paper or glass on which areas of this size have been accurately ruled out. More satisfactory results can be obtained by using circular instead of square areas. Duplicate smears should be made on the same slide. The milk is then dried with gentle heat, the fat dissolved out with xylol or other fat solvent, the smear again dried, immersed in alcohol for a few minutes to fix the film, again dried and stained with methylene blue or other stain. Alkaline stains or others which attack the casein and loosen the smear must be avoided. The glassware used must be cleansed but need not be sterilized, as the bacteria have no chance to increase in number.

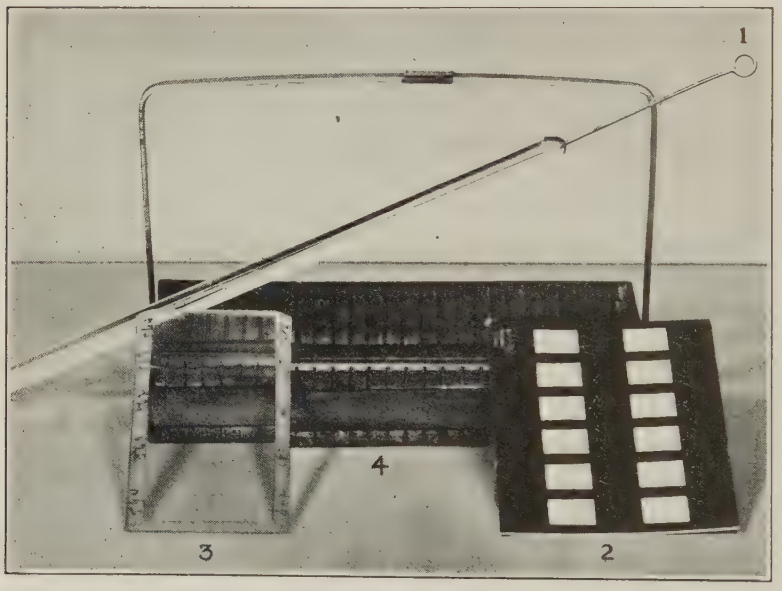


FIG. 213.—Part of outfit used for direct microscopic work. 1, loop for slide work; 2, guide; 3, slide with bevelled edges for sample number; 4, slide carrier with folding handle.

The method evolved for departmental use is a combination of what seemed to us to be the best features of the methods of our own laboratories as well as others. The outfit used for field work consists of: (1) Sterile aluminum pipettes; (2) numbered sterile sample bottles; (3) fused platinum loop taking up 0.01 c.c. of milk; (4) 1 inch straight platinum or chrome wire fitted with a handle; (5) plain glass slides, size 3.5 by 1.5 inches, with roughened bevelled edges for number of sample; (6) paper guide ruled in centimeter squares; (7) slide carrier accommo-

dating 20 to 25 slides; (8) white enamel staining dish; (9) sterno or alcohol lamp.

Method of Making Milk Smears.—Use sterile glass slides marked in centimeter squares or place the plain slide over the paper guide; mark number of sample on bevelled edge over square. Flame platinum loop, set aside to cool. Shake sample 25 times, remove cap, take up milk with sterilized cooled loop. Place in center of square marked on slide or shown by paper guide. Using sterilized short platinum needle, spread milk evenly over square. Dry in air or with gentle heat. See that smears are thoroughly dry before staining to prevent loss of smear. When dry, the slides are placed in the carrier; this is now placed in the staining dish and the staining fluid is poured gently into the dish. The carrier with the slides is kept in the staining solution for four minutes, then lifted out and placed upon folded newspapers to drain and thoroughly dry. The stain is filtered immediately and may be used quite a number of times. When dry, the carrier is placed into another enamel dish and the slides washed in running water until only a light blue color remains. The slides are dried and examined.

Formula of Newman's Stain.[1]

Methylene blue, powdered, certified	2 gm.
Ethyl alcohol, 95 per cent	60 c.c.
Xylene C.P., water-free	40 "
Glacial acetic acid	6 "

Method of Preparing Stain.—Dissolve methylene blue in warm 70 per cent alcohol, adding the powdered stain very slowly. Then add xylene and acetic acid. Filter and keep in tightly stoppered bottles.

The counting of the bacteria is done by a microscope and an oil-immersion lens. If the diameter of the field is so adjusted, by means of the draw tube, that it equals 0.16 mm., then each field of the microscope covers approximately one-five-thousandth (0.0002) of a square centimeter. On this basis each bacterium seen in a field taken at random represents 500,000 per c.c. if they are evenly distributed. However, it is impossible to distribute them evenly so that at least 10 fields of the microscope should be counted when there are many bacteria, 50 fields when the number is moderate and 100 fields if accurate results are required. The total number of bacteria seen in 10 fields multiplied by 50,000 or the total number seen in 100 fields multiplied by 5000 gives approximately the total number of bacteria per cubic centimeter. The counts vary considerably when individual bacteria are counted, even when made by the same examiner, because of the presence of one or more large clumps in one series of fields and not in another. Even when clumps are counted as one the distribution varies in the different fields.

[1] Newman, R. W.: One Solution Technique: The Direct Microscopic Count, Bull. State Dept. of Agriculture, California, 1926.

COMPARISON BETWEEN MICROSCOPIC COUNTS BY DIFFERENT EXAMINERS.

Examiner.		
A	Count of individual bacteria	164,000
	Count of clumps	65,000
B	Count of individual bacteria	29,000
	Count of clumps	8,000
C	Count of individual bacteria	133,000
	Count of clumps	38,000

The Little Plate Method.[1]—This modified plate method, while having no official standing, is sufficiently promising to justify a brief description. A sterile slide is substituted for the large plate, $\frac{1}{20}$ c.c. of undiluted milk is measured thereon, a drop of agar is added, the two are thoroughly mixed, are then spread over a 4 sq. cm. area, allowed to set and then incubated in a sterile moist chamber for a few hours. Four or five hours incubation is sufficient for the development of minute macroscopic colonies. The film is then quickly dried and, after staining, is ready for counting. The count is made under the compound microscope, which must be standardized as for the smear method given above. Comparative work by this method and by the routine plate method has given on the whole very similar results. This method also presents the advantages of (1) a greatly shortened period required for the test; (2) economy; (3) adaptability to field work; (4) examination of a larger portion of the sample, and (5) permanence of the slides for reference purposes.

Varieties.—Excluding pathogenic bacteria from human sources, bacteria in milk can be divided into two great groups—those which get into the milk after it leaves the udder and those which come from the cow. The first group comprises bacteria from dust, hands, milking pails, strainers, etc.

The number of bacteria in any sample of milk depends on three factors: the number deposited in the milk from the cow's udder, from the hands of the milker, from the air, and utensils; the time during which they have developed, and the temperature at which the milk has stood. The last is perhaps the most important factor.

The attempt was made during a period of one year to connect illness in infants and children with special varieties of saprophytic bacteria in milk, but with negative results.

From the milks examined by us altogether 239 varieties of bacteria were isolated and studied. These 239 varieties, having some cultural or other differences, were divided into 31 classes, each class containing from 1 to 39 more or less related organisms.

As to the sources of bacteria found in milk, we made sufficient experiments to satisfy us that they came chiefly from outside the udder and milk ducts.

[1] Frost, W. D.: Improved Technic for the Micro or Little Plate Method of Counting Bacteria in Milk, Jour. Inf. Dis., vol. **26**, 176. Hatfield, H. M., and Park, W. H.: A Study of the Practical Value of the Frost Little Plate Method in the Routine Colony Count of Milk Samples, Jour. Am. Pub. Health Assn., June, 1922.

Bacteria were isolated from various materials which under certain conditions might be sources of contamination for the milk, and the cultures compared with those taken from milk. Thus there were obtained from 20 specimens of hay and grass, 31 varieties of bacteria; from 15 specimens of feces, manure, and intestinal contents, 28 varieties; from 10 specimens of feed, 17 varieties. Of these 76 varieties there were 42 which resembled closely those from milk—viz., 11 from grass or hay; 26 from manure; 5 from feed.

During the investigation a number of the varieties isolated from milk were shown to be identical with types commonly found in water.

From the few facts quoted above and from many other observations made during the course of the work it would seem that the term "milk bacteria" assumes a condition which does not exist in fact.

It was found that milk taken from a number of cows, in which almost no outside contamination had occurred, and plated immediately, contained, as a rule, very few bacteria, and these were mostly streptococci. A number of specimens of fairly fresh market milk averaging 200,000 bacteria per cubic centimeter were examined immediately, and again after twelve to twenty-four hours. In almost every test the three or four predominant varieties of the fresher milk remained as the predominant varieties after the period mentioned.

The above experiments seem to show that organisms which have gained a good percentage in the ordinary commercial milk at time of sale will be likely to hold the same relative place for as long a period as milk is usually kept. After the bacteria pass the ten or twenty million counts a change occurs, since the increasing acidity inhibits the growth of some forms before it does that of others. Thus some varieties of the lactic acid bacteria can increase until the acidity is twice as great as that which inhibits the growth of many bacteria. Before milk reaches the curdling point, the bacteria may have reached over a billion to each cubic centimeter. For the most part specimens of milk from different localities showed a difference in the character of the bacteria present, in the same way that the bacteria from hay, feed, etc., varied. Even the intestinal contents of cows, the bacteriology of which might be expected to show common characteristics, contained, besides the predominating colon types, other organisms, which differed widely in different species and in different localities. Cleanliness in handling the milk and the temperature at which it had been kept were also found to have a marked influence on the predominant varieties of bacteria present.

Pathogenic Properties of the Bacteria Isolated.—The intraperitoneal injection of guinea-pigs with 2 c.c. of broth or milk cultures of about 40 per cent. of the varieties tested caused death. Cultures of most of the remainder produced no apparent deleterious effects even when injected in larger amounts. The filtrates of broth cultures of a number of varieties were tested, but only one was obtained in which poisonous products were abundantly present. Death in guinea-pigs weighing 300 grams followed within fifteen minutes after an injection of 2 c.c.; 1 c.c. had little effect.

As bacteria in milk are swallowed and not injected under the skin, it seemed wise to test the effect of feeding them to very young animals. We therefore fed forty-eight cultures of 139 varieties of bacteria to kittens of two to ten days of age by means of a glass tube. The kittens received 5 to 10 c.c. daily for from three to seven days. Only one culture produced illness or death. Very young guinea-pigs were fed in the same manner with similar results.

After five years of effort to discover some relation between special varieties of bacteria found in milk and the health of children the conclusion has been reached that neither through animal tests nor the isolation from milk given sick infants have we been able to establish such a relation. Pasteurized or "sterilized" milk is rarely kept longer than thirty-six hours, so that varieties of bacteria which, after long standing, develop in such milk did not enter into our problem. The harmlessness of cultures given to healthy young kittens does not, of course, prove that they would be equally harmless in infants. Even if harmless in robust infants, they might be injurious when summer heat and previous disease had lowered the resistance and the digestive power of the subjects.

Streptococci in Relation to Disease.—Prevalence —Frost[1] states in his summary at the end of a report:

"It will be seen that hemolytic streptococci were found in the milk of 28 per cent. of all the cows examined and that the alpha type was about twice as common as the beta type.

"It seems very evident, then, that hemolytic streptococci are to be found quite constantly in the udders of a considerable proportion of cows, even when kept under the best conditions and with frequent examinations by veterinarians and of the help by physicians. Such milks have been widely used in a raw state without causing disease and may, therefore, be looked upon as harmless. This does not mean, of course, that forms pathogenic for man may not occur in milk, but to differentiate them from the bovine forms is another question."

It is impossible to differentiate in routine milk examinations the pathogenic streptococci of diseased cows from saprophytic varieties. Thus it happens that a milk which contains great numbers of streptococci may or may not be more dangerous than one which contains an equal number of other apparently less harmful bacteria. The identification of the varieties present requires great care and is done only in the face of feared infection such as an epidemic of septic throats. Those that produce human diseases, except in infants, are probably always from cows in which the udder has been infected from human sources. If we wish to search for them, Brown, Frost and Shaw state:

"This, then, is the type of streptococcus which should be sought for as the actual or potential cause of epidemics of septic sore throat—a streptococcus of round or slightly flattened elements, producing hem-

[1] Frost, W. D., and Backman, F. M.: Hemolytic Streptococci in High-grade Milk, Jour. Am. Pub. Health Assn., 1923, **13**, 302.

olyzed zones of the beta type in blood agar, rapidly hemolyzing blood corpuscles in fluid media, markedly pathogenic for mice, fermenting glucose, lactose, saccharose and salicin, but not mannite, producing a final hydrogen-ion concentration not above pH 4.8 in glucose broth, failing to hydrolyze sodium hippurate, and probably always encapsulated when observed under suitable conditions." The streptococci having these characteristics they name *S. epidemicus*.[1] Smith and Brown[2] state: "There is no satisfactory evidence that bovine streptococci associated with mastitis or garget are the agents of tonsillitis in man."

The Deleterious Effect on Infants of Live Bacteria in Milk.—We tested this in the following way: During each of the summers of 1902, 1903, and 1904 a special lot of milk was modified for a group of fifty infants, all of whom were under nine months of age, and distributed daily. To one-half a portion of the milk was given raw; to the other half a portion heated at 60° for twenty minutes.

The modified milk was made from a fairly pure milk mixed with ordinary cream. The bacteria contained in the milk numbered on the average 45,000 per cubic centimeter, in the cream 30,000,000. The modified raw milk taken from the bottles in the morning averaged 1,200,000 bacteria per cubic centimeter, or considerably less than the ordinary grocery milk, the pasteurized about 1000; taken in the late afternoon of the same day they had, respectively, about 20,000,000 and 50,000.

Twenty-one predominant varieties of bacteria were isolated from six specimens of this milk collected on different days. The varieties represented the types of bacteria frequently found in milk. The infants were selected during the first week in June, and at first all were placed on pasteurized milk. The fifty infants which had been selected were now separated into two groups as nearly alike as possible. On the fifteenth of June the milk was distributed without heating to one-half the infants, the other half receiving as before the heated milk. In this way the infants in the two groups received milk of identically the same quality, except for the changes produced by heating to 165° F. for thirty minutes. The infants were observed carefully for three months and medical advice was given when necessary. When severe diarrhea occurred barley water was substituted for milk.

The first season's trial gave the following results: Within one week 20 out of 27 infants put on raw milk suffered from moderate or severe diarrhea while during the same time only 5 cases of moderate and none of severe diarrhea occurred in those taking pasteurized milk. Within a month 8 of the 27 had to be changed from raw back to heated milk, because of their continued illness; 7, or 25 per cent., did well all summer on raw milk. On the other hand, of those receiving the pasteurized milk, 75 per cent. remained well, or nearly so, all summer, while 25 per cent. had one or more attacks of severe diarrhea. There were no deaths in either group of cases.

[1] Jour. Infect. Dis., 1926, **38**, 381.

[2] Jour. Med. Research, 1914, **31**, 500.

During the second summer a similar test was made with 45 infants. Twenty-four were put on raw modified milk; 13 of these had serious diarrhea, in 5 of whom it was so severe that they were put back upon heated milk; 10 took raw milk all summer without bad effects; 2 died, 1 from gross neglect on the part of the mother, the other from diarrhea. Of the 21 on pasteurized milk, 5 had severe attacks of diarrhea, but all were kept on this milk except for short periods, when all food was omitted; 16 did well throughout the summer. One infant, markedly rachitic, died. The third summer's results have not been tabulated, but were similar to those of the first two tests.

The outcome of these observations during the first two summers are summarized in the following table:

Kinds of milk (number of bacteria when consumed).	Number of infants.	Remained well for entire summer.	Number having severe or moderate diarrhea.	Average number days off milk during summer.	Average weekly gain in weight, oz.	Average number of days diarrhea.	Deaths.
Pasteurized milk, 1000–50,000 bacteria per c.c.	41	31	10	3.0	4.0	3.9	1
Raw milk, 1,200,000–20,000,000 bacteria per c.c.	51[1]	17	33	5 5	3 5	11.5	2

Although the number of cases was not large, the results, almost identical during the three summers, indicate that even a fairly pure milk, when given raw in hot weather, causes illness in a much larger percentage of cases than the same milk given after pasteurization. A considerable percentage of infants, however, do quite as well apparently on raw as on pasteurized milk.

Bacteria in Milk. Effect on Older Children.—The children over three years of age who received unheated milk, containing at different times from 145,000 to 350,000,000 bacteria per cubic centimeter, showed almost no gastro-intestinal disturbance. The conditions at three institutions will serve as examples.

In the first of these an average grade of raw milk was used which, during the summer, contained from 2,000,000 to 30,000,000 bacteria per cubic centimeter. This milk was stored in an ice-box until required. It was taken unheated by children and yet no case of diarrhea of sufficient gravity to send for a physician occurred during the entire summer. This institution was an orphan asylum containing 650 children from three to fourteen years of age—viz., three to five years, 98; five to eight years, 162; eight to fourteen years, 390.

A second institution used an unheated but very pure milk which was obtained from its own farm. This milk averaged 50,000 bacteria per cubic centimeter. The inmates were 70 children of ages

[1] Thirteen of the 51 infants on raw milk were transferred before the end of the trial to pasteurized milk because of serious illness. If these infants had been left on raw milk it is believed by the writers that the comparative results would have been even more unfavorable to raw milk.

ranging from three to fourteen years. In this institution not a single case of diarrheal disease of any importance occurred during the summer.

In a third institution an average grade of milk was used which was heated. This milk before heating contained 2,000,000 to 20,000,000 bacteria per cubic centimeter. This institution was an infant asylum in which there were 126 children between the ages of two and five years. There were no cases of diarrhea during the summer.

These clinical observations taken in connection with the bacteriological examination at the laboratory show that although the milk may come from healthy cattle and clean farms and be kept at a temperature not exceeding 60° F., a very great increase in the number of bacteria may occur. Furthermore, this may occur without the accumulation in the milk of sufficient poisonous products or living bacteria to cause appreciable injury in children over three years of age, even when such milk is consumed in considerable amount and for a period extending over several months. Milk kept at a temperature somewhat above 60° F. was not met with in our investigations, but the histories of epidemics of ptomain poisoning teach that such milk may be very poisonous. It is also to be remembered that milk abounding in bacteria on account of careless handling is always liable to contain pathogenic organisms derived from human or animal sources.

Results with Very Impure Milk Heated vs. Those with Pure or Average Milk Heated.—During the summer of 1901 we were able to observe a number of babies fed on loose milk grossly contaminated by bacteria. In 1902 systematic supervision of all stores selling milk was instituted by the Health Department, so that the very worst milk was not offered for sale that summer.

The observations upon the impure milk of 1901 are of sufficient importance to be given in detail, although already mentioned in the report of the observations upon infants of both summers which were fed on "store milk." A group of over 150 infants was so divided that 20 per cent. were allowed to remain on the cheapest store milk which they were taking at the time. To about the same number was given a pure bottled milk. A third group was fed on the same quality of milk as the second, but sterilized and modified at the Good Samaritan Dispensary. A fourth group received milk from an ordinary dairy farm. This milk was sent to a store in cans and called for by the people. A few infants fed on breast and condensed milk were observed for control.

In estimating the significance of the observations recorded in the tables, one should bear in mind that not only do different infants possess different degrees of resistance to disease, but that, try as hard as the physicians could, it was impossible to divide the infants into groups which secured equal care and were subjected to exactly the same conditions. It was necessary to have the different groups in somewhat different parts of the city. It thus happened that the infants on the cheap store milk received less home care than the average, and that those on the pure bottled milk lived in the coolest portion of the

city. Certain results were, however, so striking that their interpretation is fairly clear. It is to be noted that the number of infants included in each group is small.

TABLE SHOWING THE RESULTS OF FEEDING DURING JULY AND AUGUST, 1901, IN TENEMENT HOUSES, OF 112 BOTTLE-FED INFANTS UNDER ONE YEAR OF AGE, AND OF 47 BOTTLE-FED INFANTS BETWEEN ONE AND TWO YEARS OF AGE WITH MILK FROM DIFFERENT SOURCES, AND THE NUMBER OF BACTERIA PRESENT IN THE MILK.

Character of milk.	Infants under one year.					Infants over one year.				
	Number of infants.	Average weekly gain.	Diarrhea.		Deaths.	Number of infants.	Average weekly gain.	Diarrhea.		Deaths.
			Mild.	Severe.				Mild.	Severe.	
		oz.					oz.			
1. Pure milk boiled and modified at dispensary or stations; given out in small bottles; milk before boiling averaged 20,000 bacteria per c.c.; after boiling 2 per c.c.	41	3	10	8	1[1]					
2. Pure milk, twenty-four hours old, sent in quart bottles to tenements, heated and modified at home. 20,000 to 200,000 bacteria per c.c. when delivered.	23	4⅛	8	5	0	24	4½	8	2	0
3 Ordinary milk, thirty-six hours old, from a selected group of farms, kept cool in cans during transport; 1,000,000 to 25,000,000 bacteria per c.c., heated and modified at home before using.	18	4	6	6	1[2]	12	4	1	2	0
4. Cheap milk, thirty-six to sixty hours old, from various small stores, derived from various farms, some fairly clean, some very dirty; 400,000 to 175,000,000 bacteria per c.c. before home heating.	21	¼	4	13	4[3]	7	⅓	1	3	0
5. Condensed milk of different brands; made up with hot water; as given, contained bacteria from 5000 to 200,000 per c.c.	9	¼	5	2	3	4	3⅛	1	3	0
6. Breast milk	16	2¼	5	2	0					

[1] This infant died from enteritis and toxemia.
[2] This infant died of pneumonia. There had been no severe intestinal disorder noted.
[3] One of the four had pertussis, the remaining three died from uncomplicated enteritis.

There is nothing in the observations to show that fairly fresh milk from healthy cows, living under good hygienic conditions and containing, on some days, when delivered, as many as 200,000 bacteria per cubic centimeter, had any bacteria or any products due to bacteria that remained deleterious after the milk was heated to near the boiling-point.

On the other hand, it is possible that certain varieties of bacteria may, under conditions that are insanitary, find entrance to milk and survive moderate heat or may develop poisonous products resistant

to heat in sufficient amount to be harmful, even when they have accumulated to less than 200,000 per c.c.

Turning now to the results of feeding with milk which has been heated and which before sterilization contained from 1,000,000 to 25,000,000 bacteria per cubic centimeter, averaging about 15,000,000, though obtained from healthy cows living under fairly decent conditions and although the milk was kept moderately cool in transit, we find a distinct increase in the amount of diarrheal diseases. Though it is probable that the excessive amount of diarrhea in this group of children was due to bacterial changes which were not neutralized by heat or to living bacteria which were not killed, yet it is only fair to consider that the difference was not very great and that the infants of this group were under surroundings not quite so good as those on the pure milk.

Finally, we come in this comparison to the infants who received the cheap store milk after heating. This milk had frequently to be returned because it curdled when boiled, and contained, according to the weather, from 4,000,000 to 200,000,000 bacteria per cubic centimeter. In these infants the worst results were seen. This is shown not only by the death-rate, but by the amount and by the severity of the diarrheal diseases, and the general appearance of the children as noted by the physicians. Although the average number of bacteria in the milk received by this group is higher than that received by the previous group, the difference in results between this group and the previous one can hardly be explained by the difference in the number of bacteria. The varieties of bacteria found in this milk were more numerous than in the better milk, but we were unable to prove that they were more dangerous. Probably the higher temperature at which the milk was kept in transit, and the longer interval between milking and its use, allowed more toxic bacterial products to accumulate.

Bacterial Contamination of Milk—General Conclusions[1] as to Relative Importance.—1. During cool weather neither the mortality nor the health of the infants observed in the investigation was appreciably affected by quality of the market milk or by the number of bacteria which it contained. The different grades of milk varied much less in the amount of bacterial contamination in winter than in summer, the store milk averaging only about 750,000 bacteria per cubic centimeter.

2. During hot weather, when the resistance of the children was lowered, the kind of milk taken influenced both the amount of illness and the mortality; those who took condensed milk and cheap store milk did the worst, and those who received breast milk, pure bottled milk, and modified milk did the best. The effect of bacterial contamination was very marked when the milk was taken without previous heating; but, unless the contamination was very excessive, only slight when heating was employed shortly before feeding.

[1] These conclusions were drawn up by the authors in association with Dr. L. E. Holt, after a joint study of the results obtained in the studies above recorded.

3. The number of bacteria which may accumulate before milk becomes noticeably harmful to the average infant in summer differs with the nature of the bacteria present, the age of the milk, and the temperature at which it has been kept. When the milk is taken raw, the fewer the bacteria present the better are the results. Of the usual varieties, over 1,000,000 bacteria per cubic centimeter are certainly deleterious to the average infant. However, many infants take such milk without apparently harmful results. Heat of 145° F. for thirty minutes or of 170° F. for a shorter period not only destroys most of the bacteria present, but, apparently, some of their poisonous products. No harm from the bacteria previously existing in recently heated milk was noticed in these observations unless they had amounted to many millions, but in such numbers they were decidedly deleterious.

4. When milk of average quality was fed, pasteurized and raw, those infants who received milk previously heated did, on the average, much better in warm weather than those who received it raw. The difference was so quickly manifest and so marked that there could be no mistaking the meaning of the results.

5. After the first twelve months of life infants are less and less affected by the bacteria in milk derived from healthy cattle and from the air. According to these observations, when the milk had been kept cool, the bacteria, unless in very great excess, did not appear to injure the children over three years of age at any season of the year.

The Bactericidal Property of Cows' Milk.—Hesse, in 1894, noted that the growth of *B. typhosus* was inhibited in raw milk. One of the writers noted in 1895 that the number of bacteria decreased for a time in fresh milk. Jones and Little[1] have made a study of this property. They found that the milk of all the cows examined inhibited the growth of their test organism, the non-hemolytic mastitis streptococcus. With different milks the inhibition lasted from four to eight hours. They infer that the substance originates in the udder and is not specific.

Influence of Temperature upon the Multiplication of Bacteria in Milk.—Few, even of the well informed, appreciate how great a difference a few degrees of temperature will make in the rate of bacterial multiplication. Milk rapidly and sufficiently cooled keeps almost unaltered for thirty-six hours, while milk insufficiently cooled deteriorates rapidly.

The majority of the bacteria found in milk grow best at temperatures above 70° F., but they also multiply slowly even at 40° F.; thus, of 60 species isolated by us, 42 developed good growth at the end of seven days at 39° F. Our observations have shown that the bacteria slowly increase in numbers after the germicidal properties of the milk have disappeared and the germs have become accustomed to the low temperature. In fact, milk cannot be permanently preserved unaltered unless kept at 32° F. or less. The degree of cooling to which ordinary supplies of milk are subjected differs greatly in various localities. Some farmers chill their milk rapidly, by means of pipe coils over which the milk flows; others use deep wooden tanks filled with water into which

[1] Jour. Exper. Med., 1927, **45**, 319.

the cans of milk are placed soon after milking. In winter these methods are very satisfactory, for the water runs into the pipes or tanks at about 38° F. In warmer weather they are unsatisfactory, unless ice is used, as the natural temperature of the water may be as high as 55° F. A considerable quantity of milk is not cooled at all at the farms. It is sent to the creamery or railroad after two to six hours, and is then more or less cooled. These few hours in summer, when the milk is left almost at blood heat, allow an enormous development of bacteria to take place, as is shown in the table below.

TABLE I.—SHOWING THE DEVELOPMENT OF BACTERIA IN TWO SAMPLES OF MILK MAINTAINED AT DIFFERENT TEMPERATURES FOR TWENTY-FOUR, FORTY-EIGHT, AND NINETY-SIX HOURS, RESPECTIVELY. THE FIRST SAMPLE OF MILK WAS OBTAINED UNDER THE BEST CONDITIONS POSSIBLE, THE SECOND IN THE USUAL WAY. WHEN RECEIVED, SPECIMEN NO. 1 CONTAINED 3000 BACTERIA PER C.C., SPECIMEN NO. 2, 30,000 PER C.C.

Temperature. Fahrenheit.	Time which elapsed before making tests. 24 hours.	48 hours.	96 hours.	168 hours.
32°	2,400	2,100	1,850	1,400
	30,000[1]	**27,000**	**24,000**	**19,000**
39°	2,500	3,600	218,000	4,209,000
	38,000	**56,000**	**4,300,000**	**38,000,000**
42°	2,600	3,600	500,000	11,200,000
	43,000	**210,000**	**5,760,000**	**120,000,000**
46°	3,100	12,000	1,480,000	80,000,000
	42,000	**360,000**	**12,200,000**	**500,000,000**
50°	11,600	540,000	300,000,000	
	89,000	**1,940,000**	**5,000,000,000**[2]	
55°	18,800	3,400,000		
	187,000	**38,000,000**		
60°	180,000	28,000,000		
	900,000	**168,000,000**		
68°	450,000	500,000,000		
	4,000,000	**10,000,000,000**[2]		

OBSERVATIONS ON BACTERIAL MULTIPLICATION IN MILK AT 90° F., A TEMPERATURE OCCURRING IN NEW YORK IN HOT SUMMER WEATHER.

TABLE II.—NUMBER OF BACTERIA PER C.C.

	Milk I. Fresh and of good quality.	Milk II. Fair quality from store.	Milk III. Bad quality from store.
Original number	5,200	92,000	2,600,000
After two hours	8,400	184,000	4,220,000
After four hours	12,400	470,000	19,000,000
After six hours	68,500	1,260,000	39,000,000
After eight hours	654,000	6,800,000	124,000,000

A sample of milk No. 1 removed after six hours and cooled to 50° F. contained 145,000,000 at the end of twenty-four hours. Some of this milk, kept cool from the beginning, contained but 12,800 bacteria per cubic centimeter at the end of twenty-four hours.

Pasteurization of Milk.—The two dominant factors which control the time and temperature at which the milk should be heated are: (1) The thermal death-points of pathogenic bacteria; (2) the thermo-

[1] The figures referring to tests of the second sample are printed in heavy-face type.
[2] These figures signify the maximum growth and are conservative estimates only.

labile food constituents of the milk; and (3) the effect on the cream line. The first factor is almost equally important for milk used by persons of all ages, while the second factor is important only for milk used for very young children. The third factor is important because it is desirable to make milk as attractive a food as possible.

The exposure of bacteria for a short time at a high temperature is equivalent to a longer time at a lower temperature. The ferments and other labile food constituents, on the other hand, are altered much more by the higher temperature. It is well, therefore, to choose the lowest possible temperature which will kill the non-spore-bearing pathogenic bacteria in a practicable length of time. Such an exposure is 60° C. (140° F.) for twenty minutes, 70° C. (158° F.) for two minutes. Temperatures below 146° F. alter the cream separation only slightly. Very much shorter exposures, as one minute at 70° C., will kill a large majority of pathogenic and other bacteria in milk and greatly increase its safety as seen in the tables below, but it is wiser to take no chances.

TABLE SHOWING EFFECT OF HEAT UPON TUBERCLE BACILLI IN MILK.

Degree of heat.	Time exposed.	Amount of milk.	Result in guinea-pigs.
60° C.	15.0 min.	1 c.c.	Infection.
60° C.	20.0 min.	1 c.c.	No infection.
60° C.	30.0 min.	1 c.c.	No infection.
70° C.	0.5 min.	1 c.c.	Infection.[1]
70° C.	1.0 min.	1 c.c.	No infection.
70° C.	2.0 min.	1 c.c.	No infection.
Control not heated		0.001	Infection.

This milk was infected by adding one-fifth of its quantity of sputum rich in tubercle bacilli.

Where claims are made that 140° F. for thirty minutes does not always result in effectual pasteurization, we have shown by intensive work carried out on a commercial scale, that such findings have resulted from a faulty construction of the pasteurizing apparatus, which allowed a portion of the milk to pass without its having been treated for the required time. In order to allow for slight faults in the apparatus or in the running of the plant, most municipalities require a minimum temperature of 143° or 143.5° F. for thirty minutes.

The Sterilization of Milk for Infant Feeding.—Complete sterilization destroys all the germs in milk, and so, if no new ones gain entrance, permanently prevents fermentative changes. This requires boiling for fifteen to forty-five minutes on two or three consecutive days, according to the presence or absence of certain spores.

Milk is best sterilized by heat, for nearly all chemicals, such as boric acid, salicylic acid, and formalin, are not only slightly deleterious themselves but also make the milk less digestible, and therefore less fit for food. Formalin is the least objectionable of the three. Milk may be sterilized at a high or low temperature, that is, by boiling or at a lower degree of heat, obtained by modifying the steaming process. Milk heated to 100° C. is not altogether desirable for prolonged use for

[1] Most of the guinea-pigs were not infected by the milk heated for one-half minute.

infants, as the high temperature causes certain changes in the milk which make it less suitable as a food for them.

Pasteurization for Infant Feeding.—For home pasteurization the following is a simple and practical method.

Apparatus Required.—(*a*) A tin pail or pot, about 10 inches deep by 9 inches in diameter, provided with the ordinary tin cover which has been perforated with 8 holes each 1 inch in diameter.

(*b*) A wire basket, with eight nursing bottles (as sold for this purpose in the shops).

(*c*) Rubber stoppers for bottles and a bristle brush for cleaning the bottles.

Directions (Koplik).—Place the milk, pure or diluted (as the physician may direct), in the nursing bottles and place the latter in the wire basket. Put only sufficient milk for one nursing in each bottle. Do not cork the bottles at first.

Having previously poured about two inches of water in the tin pail or pot and brought it to the boiling-point, lower the basket of nursing bottles slowly into the pot. Do not allow the bottles to touch the water or they will crack. Put on the perforated cover and let the steaming continue for ten minutes; then remove the cover and firmly cork each bottle. After replacing the cover, allow the steaming to continue for fifteen minutes. The steam must be allowed to escape freely or the temperature will rise too high.

The process is now complete. Place the basket of bottles in a cool, dark place or in an ice-chest. The bottles must not be opened until just before using and then each may be warmed as needed by plunging it into warm water. If properly prepared the milk will have little or no boiled taste.

The temperature attained under the conditions stated above will not exceed in extreme cases 87° C. (188° F.).

A different but admirable method is the one devised by Dr. Freeman. Here a pail is filled to a certain mark with water, and then placed on the stove until the water boils. It is then removed, and immediately a milk-holder, consisting of a series of zinc cylinders, is lowered with its milk bottles partially full of milk. The cover is again applied. In ten minutes the heat of the outside water raises the temperature of the milk to about 65° C. (149° F.), and holds it at nearly that point for some time. After twenty minutes the milk is removed, placed in cold water, and quickly chilled. The milk is kept in the ice-chest until used. When milk is pasteurized in great quantities it should always be done by the "holding process," as "flash" pasteurization is unreliable. Milk should be pasteurized while as fresh as possible, and only sufficient milk for twenty-four hours should be pasteurized at one time. If, after nursing, the infant leaves some milk in the bottle this should be thrown away.

Care of the Bottles.—Nursing bottles, after use, should be filled with a strong solution of washing soda, allowed to stand twenty-four hours, and then carefully cleaned with a bristle (bottle) brush. The rubber

stoppers and nipples, after using, should be boiled in strong soda solution for fifteen minutes and then rinsed and dried.

Milk, after sterilization, should never be put into unsterilized bottles as this will spoil it.

Development of Bacteria in Milk which had been Heated.—There is a common idea that bacteria develop much more rapidly in milk that has been heated than in raw milk. This is true only for freshly drawn milk which has slight bactericidal power.

The table below shows the effect on bacteria in milk of heating to 71° C. for one-half and one minute. Not only the immediate reduction in number is seen to be great, but the difference continues when the milk is kept cold for two days.

TWO SAMPLES MIXED FROM 100 SAMPLES FROM INSPECTORS. PASTEURIZED AT 160° F. PLATES MADE SAME DAY.

Sample I.		Sample II.	
Raw milk	600,000	Raw milk	5,400,000
½ minute pasteurized	2,000	½ minute pasteurized	7,400
1 minute pasteurized	1,000	1 minute pasteurized	600
SAME SAMPLES KEPT IN ICE-BOX TWENTY-FOUR HOURS AT 45° F. (7° C.).			
Raw milk	6,300,000	Raw milk	21,600,000
½ minute pasteurized	18,000	½ minute pasteurized	12,000
1 minute pasteurized	900	1 minute pasteurized	3,600
IN ICE-BOX FORTY-EIGHT HOURS AT 45° F. (7° C.).			
Raw milk	16,200,000	Raw milk	63,000,000
½ minute pasteurized	120,000	½ minute pasteurized	276,000
1 minute pasteurized	10,000	1 minute pasteurized	90,000

Thermophilic Bacteria in Pasteurized Milk.—For years it has been known that thermophilic bacteria from the soil or elsewhere might get into milk and resist pasteurization. Lately certain pasteurizing plants found that as the run continued the number of live bacteria increased. This was found in different places to be due to different thermophilic bacteria. The most frequent were spore-bearing bacilli, but in other cases micrococci, streptococci or non-spore-bearing bacilli were present. Often when the milk run began the supply had a few thousands, while after six to twelve hours there would be hundreds of thousands or even one or two millions. Careful cleanliness and the interruption of the run served to prevent a great increase. These bacteria are not harmful, but they sometimes make the milk less desirable in its taste or appearance. The streptococci and micrococci are apt to form pin-point colonies. For further knowledge and bibliography, consult Martha Eckford[1] and Margaret Doehm.[2]

NUMBER OF BACTERIA IN MILK PRODUCED UNDER DIFFERENT CONDITIONS.

1. The number of bacteria present at the time of milking and twenty-four, orty-eight, and seventy-two hours afterward in milk obtained and kept under correct conditions.

No preservatives were present in any of the following specimens:

Pure milk obtained where every reasonable means was taken to ensure clean-

[1] Am. Jour. Hyg., 1927, **7**, 201.

[2] Jour. Infect. Dis. 1929, **45**, 61.

liness. The long hairs on the udder were clipped; the cows roughly cleaned and placed in clean barns before milking; the udders were wiped off just previous to milking; the hands of the men were washed and dried; the pails used had small (six-inch) openings, and were thoroughly cleaned and sterilized by steam before use. Milk cooled within one hour after milking to 45° F., and subsequently kept at that temperature. The first six specimens were obtained from individual cows; the last six from mixed milk as it flowed at different times from the cooler. Temperature of barns 55° F.

NUMBER OF BACTERIA IN 1 C.C. OF MILK.

FROM SIX INDIVIDUAL COWS.

	After milking.	After 24 hours.	After 48 hours.
	500	700	12,500
	700	700	29,400
	19,900	5,200	24,200
	400	200	8,600
	900	1,600	12,700
	13,000	3,200	19,500
Average	6,000	1,933	17,816

FROM SAMPLES OF MIXED MILK OF ENTIRE HERD.

	6,900	12,000	19,800
	6,100	2,200	20,200
	4,100	700	7,900
	1,200	400	7,100
	6,000	900	9,800
	1,700	400	8,700
Average	4,333	2,766	10,583

Twenty-five samples taken separately from individual cows on another day and tested immediately averaged 4550 bacteria per cubic centimeter and 4500 after twenty-four hours. These twenty-five specimens were kept at between 45° and 50° F.

2. Milk taken during winter in well-ventilated, fairly clean, but dusty barns. Visible dirt was cleaned off the hair about the udder before milking. Milkers' hands were wiped off, but not washed. Milk pails and cans were clean, but the straining cloths dusty. Milk cooled within two hours after milking to 45° F.

NUMBER OF BACTERIA IN 1 C.C. OF MILK.

	At time of milking.	After 24 hours.	After 48 hours.
	12,000	14,000	57,000
	13,000	20,000	65,000
	21,500	31,000	106,000
Average	15,500	21,666	76,000

NUMBER IN CITY MILK.

3. The condition of the average raw city milk is very different.

The results of the examination of many thousands of specimens indicate that as much as 25 per cent. of the milk produced during the summer for New York City contains numbers of bacteria before pasteurization in excess of the standards.

It must be kept in mind that milk containing moderately high numbers of bacteria per cubic centimeter will, when kept at the temperature common in the homes of the poor who comprise the larger part of the population, soon contain very largely increased numbers and show its dangerous condition by turning sour and curdling.

Cleanliness Used in Obtaining Milk, and its Influence.—It cannot be regarded as an unnecessary refinement to ask that farmers should thoroughly clean the floors of their stalls once each day, that no sweeping should be done just before milking, and that the udders should be wiped with a clean damp cloth and the milkers should thoroughly wash and wipe their hands before commencing milking. The pails and cans should not only be carefully cleansed, but afterward scalded out with boiling water. The washing of the hands would lessen the number of ordinary filth bacteria in the milk, and diminish risk of transmitting to milk human infectious diseases, like scarlet fever, diphtheria, and enteric fever, by the direct washing off of the disease germs from infected hands.

The committee on the bacteriological examination of milk of the A. P. H. A. at the meeting at Cincinnati in October, 1916, presented the following statement of the interpretation to be placed on milk counts and the number of bacteria permissible.

1. Where the analysis can be made immediately after the milking the number of bacteria enables conclusions to be drawn as to the cleanliness and care in the dairy and the thoroughness in the cleaning and sterilization of the milk vessels, or sometimes the presence of cows with diseased udders. With properly cleaned and sterilized milk vessels and proper care in the farm and dairy the number of bacteria should not exceed 10,000, and easily may be brought down to 5000. Numbers beyond these, in milk analyzed immediately after the milking, may be regarded as an indication of unclean dairy methods, dirty and unsterile milking vessels, or of diseased udders. Apart from diseased udders the factors in dairying that most noticeably increase the bacteria count are unclean udders, milking with wet hands, unsterile milk vessels, unsterile strainers, and failure to cool the milk promptly.

2. If the milk is properly cooled with ice the numbers should not increase materially in five to seven hours. Communities within five to seven hours of their dairies should be able therefore to obtain milk with nearly as low a count as above indicated. Hence in such communities bacterial counts above these numbers should not be found in properly guarded milk. A count of 50,000 in such a community is an indication either of unsatisfactory dairy conditions or of failure to properly cool the milk during transportation. Night's milk also, if properly cooled, can easily be brought within these limits if analyzed the next morning. A count of over 50,000 for a community close to the dairies must be regarded as unsatisfactory, and the number should not be much more than 10,000 for high grade milk. In hot summer weather the difficulties of keeping low counts are greater, but even then they need not exceed 30,000 if the milk is properly cooled.

3. Where milk must be a longer time in transportation from the dairy there will be inevitably an increase in bacteria, depending on the length of time and the temperature. Experience has shown, however, that even in these conditions the excessively high numbers that have frequently been found in city milk are in reality due to dirty dairy conditions, to dirty and unsterile milk utensils, or culpable neglect in cooling. Moreover, such high bacterial counts at the shipping station are frequently traceable to a few dirty dairies whose milk with an abnormally high count contaminates the rest of the supply. Dirty shipping cases and warm temperatures in shipping are responsible for most of the high bacterial counts in city milk. Where the milk from healthy cows reaches the city within twenty-four hours, however, the number should not be over 100,000 in winter or 200,000 in summer, and numbers in excess of this may be regarded as due either to improper dairy conditions, dirty milk vessels, or insufficient cooling. In larger cities where much of the milk is forty-eight hours in reaching the city higher numbers may naturally be expected. But even under these conditions there is no good reason why the number of bacteria should reach 1,000,000; and most of it can be brought below 200,000. In such cities, therefore, milk with more than 1,000,000 bacteria must be looked upon as improperly guarded either at the dairy or in transit.

4. For a Grade A milk higher demands should be made than for the ordinary grade. The standard set by the Milk Commission for Grade A, viz., of 200,000 for milk to be subsequently pasteurized or for 100,000 to be used raw, is stated by that Commission to be an extreme limit for the most unfavorable conditions. Communities favorably situated near the supplying dairies should demand a much higher standard for Grade A milk and should insist upon a bacterial content *not exceeding* 10,000.

5. For communities situated where ice is not available it may be necessary to accept a milk with a higher bacterial content; but as rapidly as possible the standard should be made to approach the limits given above.

The question might be raised, Are even these enormous numbers of bacteria often found in milk during hot weather harmful?

Our knowledge is probably insufficient as yet to state just how many bacteria must accumulate to make them noticeably dangerous in milk. Some varieties are undoubtedly more harmful than others, and we have no way of restricting the kinds that will fall into milk, except by enforcing cleanliness. We have also to consider that milk is not entirely used for some twelve hours after being purchased, and that during all this time bacteria are rapidly multiplying, especially where, as among the poor, no provision for cooling it is made. Slight changes in the milk which to one child would be harmless, would in another produce disturbances which might lead to serious disease. A safe conclusion is that no more bacterial contamination should be allowed than it is practicable to avoid. Any intelligent farmer can use sufficient cleanliness and apply sufficient cold, with almost no

increase in expense, (½ cent per quart), to supply milk twenty-four to thirty-six hours old which will not contain in each cubic centimeter over 50,000 to 100,000 bacteria, and no milk containing more bacteria should be sold.

The most deleterious changes which occur in milk during its transportation are now known to be due not to skimming off the cream or to the addition of water, but to the changes produced in the milk by multiplication of bacteria. During this multiplication, acids and distinctly poisonous bacterial products are added to the milk to such an extent that much of the milk becomes distinctly deleterious to infants and invalids. It is the duty of health authorities to prevent the sale of milk rendered unfit for use because of excessive numbers of bacteria and their products.

The culture tests to determine the number of bacteria present in any sample of milk require at least forty-eight hours; so that the sale of milk found impure cannot be prevented. It will, however, be the purpose of the authorities gradually to force the farmers and the middlemen to use cleanliness, cold, and dispatch in the handling of their milk, rather than to prevent the use of the small amount tested on any one day.

If the milk on the train or at the dealer's is found to contain excessive numbers of bacteria, the farmers will be cautioned and instructed to carry out the simple necessary rules furnished them on a printed form.

Transmission of Contagious Diseases through Milk.—Pathogenic Bacteria in Milk.—Tuberculosis, typhoid and paratyphoid fever, septic sore-throat, scarlet fever, diphtheria and undulant fever, derived from cows' milk in the Northern States and from both cows' and goats' in the Southern States (when derived from goats' milk it is usually called Malta fever) are the chief diseases transmitted by means of milk in this country. In other countries cholera, dysentery and possibly other diseases may be due at times to milk infection.

The tubercle bacilli are in the majority of cases derived from the cow, but may come from human sources, the typhoid bacilli are entirely from man, the contagion of true scarlet fever and of septic throat, which may closely simulate scarlet fever, are due to hemolytic streptococci probably of human origin, infecting the milk either directly from a human source or indirectly after intermediate infection of the cow's udder. Even when no appreciable inflammation can be detected[1] the milk from one or more quarters may be richly seeded with streptococci. Diphtheria bacilli are probably always of human origin, as animals, except cats, practically never suffer from the disease, and these only under exceptional conditions. Slight ulcerations on the teats may become infected and so seed the milk. As milk is usually kept below 60° F., the typhoid bacilli and the streptococci are the only pathogenic germs that we believe increase to any appreciable extent.

[1] Krumwiede and Valentine: Jour. Med. Res., 1915, **33**, 231. White: Historical Review, New England Jour. Med., 1929, **200**, 797.

The following epidemics and cases have been recorded in the bulletin of the Public Health Service, as produced by unheated cow's milk:

	Epidemics.	Cases.
Typhoid fever	179	6900
Scarlet fever	51	2400
Septic sore-throat	7	1100
Diphtheria	23	960

Tuberculosis. See chapter on tuberculosis.

No case of measles, smallpox, chicken-pox, whooping-cough or mumps has been clearly traced to milk, but several cases of poliomyelitis have been traced to it by Aycock and Godfrey.

The Relation of the Typhoid Carrier to Milk Infection.—Many epidemics of typhoid fever formerly puzzled investigators because, though evidently milk-borne, no case of typhoid fever could be found. The discovery that about 2 per cent. of those who have recovered from typhoid fever remain infected and continue during the rest of their lives to pass typhoid bacilli has cleared up the mystery. Epidemics due to these carriers have already been traced, both in New York City and elsewhere. Many observers have already discussed the relation of typhoid cases to milk infection. Hands, water, flies, etc., may all aid in the transfer of the bacilli from the dejecta to the milk. The year before pasteurization was made compulsory we traced over 400 cases to infection of a milk supply by a typhoid carrier who had the disease forty-seven years ago.

The Conveyance of Scarlet Fever by Means of Milk. We know that the throat, nose and ear discharges are dangerous. Where the infection has been traced it has usually been found that the milker has suffered from an unrecognized case or is convalescent. A small number of epidemics have appeared to come from the milk of diseased cows. Many are skeptical about this, but after personal experience we think it probable. The history of one case was as follows: The milk from a septic cow was delivered to two schools. About thirty of the boys who drank the milk developed the disease while none of the day scholars who went home to lunch did. Some of the cases developed at first only sore-throats, others only the rash. On the second day the cases resembled scarlet fever. There was no scarlet fever in the town. The milk contained immense numbers of long-chained hemolytic streptococci. We did not determine whether this streptococcus belonged to the group of human scarlet fever strains.

The Grading of Milk.—The appreciation of the importance of the bacteria in milk has led to the pasteurization of all milk entering most large cities, except that produced and transported under the very best conditions. Together with the farm conditions the bacterial content of milk is used to grade it. The Public Health Service has developed an excellent system of grading which allows several grades of both raw and certified. In this way any community can adopt it, allowing those grades which at the time it is necessary to have. All milk in New York City is divided into grades A and B.

Grade A is raw and pasteurized. The raw is from cows which have successfully passed the tuberculin test. It must be certified by a Medical Milk Commission.

Grade B is all pasteurized.

Grade C in many communities is allowed.

The grade A may contain: Certified 10,000, raw to be pasteurized 100,000 and after pasteurization 30,000 bacteria per cubic centimeter.

The grade B may contain 1,500,000 when raw and 100,000 after pasteurization.

The grade C may contain any number within reason before and after pasteurization. It is supposed to be used for cooking purposes only and must be pasteurized or boiled. The State of New York has adopted a similar grading except that it allows raw and pasteurized in all three grades. Many cities and states are adopting such grades. Grading not only permits the consumer to know the sanitary quality of the milk, but it tends to provide the best milk possible, since the higher grades obtain a higher value.

CHAPTER XLIX.

THE BACTERIOLOGICAL EXAMINATION OF SHELLFISH.

Of the shellfish used for food, lobsters, crabs, shrimp, oysters, clams, mussels, etc., oysters and clams are the most important from the standpoint of disease production because they are so commonly consumed uncooked.

Oysters and clams when growing in waters not contaminated by human or animal pollution are free of dangerous bacteria. With the general and growing sewage pollution of tidal waters many areas have become, or are becoming, unsuited because of the dangers of contamination by fecal pathogens, especially B. typhosus.

There is a seasonal variation in the physiological activity of the oyster. During the warmer months the oyster is very active, passing large quantities of water, "drinking," and has at this time a relatively high content of bacteria. When the temperature falls, activity is less, the shells closing at about 45° F. and usually remaining closed at lower temperatures. The oyster then passes no water and the bacterial content falls very much. This inactive condition Pease has termed hibernation.

The total number of bacteria, including pathogens (and therefore the B. coli score—see below), fall rapidly after hibernation ensues. This condition may be looked upon as a relative safeguard. It is, insofar as hibernation will tend to prevent contamination of the interior of the oyster. It is also a relative safeguard in that the numbers of dangerous bacteria will decrease, but it is no absolute safeguard as B. typhosus will persist in hibernating oysters much longer than in oysters which are actively drinking. Krumwiede, Park and their co-workers[1] have isolated B. typhosus from oysters fifty-one days after contamination and return to natural waters of low temperature (December, January).

The transfer of oysters from polluted waters to clean waters will result in a marked and rapid drop of the numbers of bacteria, if the oysters drink freely in the clean waters. This procedure has been recommended for the "purification" of oysters. We[2] have restudied this problem recently and found that if oysters contaminated with B. typhosus are planted in natural waters during the warm months, B. typhosus cannot be isolated after fourteen days. In sea water B. typhosus may survive in small numbers for nineteen to twenty-four days. Evidently this procedure cannot be relied upon to render oysters safe and especially not during the colder months when drinking after transfer would be relatively less, or even absent. The Health Department, New York city allows the transfer of oysters from doubtful beds to clean waters

[1] Am. Jour. Public Health, 1926, **16**, 263.
[2] Ibid., 1928, **18**, 48.

only during the closed season. The waters to which transfer is made may not be fished for thirty days after transfer. Conditioning of oysters and wet storage should only be allowed where pure water of proper salinity is available.

Wells has recommended the use of chlorinated sea water in tanks as a means of purifying oysters. When the chlorine content falls sufficiently the oysters are supposed to drink this purified water and thus cleanse themselves. A second chlorination is then done to kill the excreted bacteria and the oysters again allowed to drink after the chlorine content falls. Naturally the temperature of the water used is an important factor as regards drinking.

We (Krumwiede, Park and co-workers)[1] have found that the method, although it may reduce the numbers, will not kill all the B. typhosus on the shell of contaminated oysters nor eliminate all the B. typhosus from the liquor or oyster body; even though the amount of chlorine and the numbers of drinkings be much more than recommended by Wells. To speak of the use of chlorinated water as a purification method, therefore, is not fully warranted. Chlorination, however, is to be recommended as a means of obtaining a pure and safe water for the storage of oysters.

After collection, oysters may be stored, especially when shipped inland. If they are contaminated with B. typhosus, residual numbers may outlive the oyster. Should a contaminated oyster die and should the temperature be suitable, multiplication will take place and the drippings from the dead opened oyster may be a source of recontamination of the other oysters.

Oysters and clams are shucked and shipped to market in containers. Such stock is usually used for cooking. In the process of preparation the oysters are washed and then freed of as much liquid as possible and stocked in cans which are preferably not returnable. Unless this process is carried out in a most cleanly manner the bacterial count and B. coli score will be higher than that of the unshucked stock. This will be followed by a further rise if the shucked stock is not adequately refrigerated. A small amount of a thick mucoid liquid is present in the containers of shucked oysters. B. typhosus will multiply in this if the temperature is satisfactory.

Oysters have been the source of epidemics and of individual cases of typhoid. There is one important element in the histories of oyster-borne outbreaks: nearly all have been traced to the contamination of the oysters after dredging, that is, through contamination during drinking or storage. One recent small outbreak was traced to an oysterman who is a chronic carrier. Such evidence indicates a considerable natural safeguarding of oysters in their beds even where some sewage pollution occurs. On the other hand all the actual oyster-borne cases of typhoid are not traced. If we knew the actual total numbers and the conditions, the apparent natural safeguard might not appear so real.

[1] Am. Jour. Public Health, 1926, **16**, 142.

In determining whether or not oysters or clams may be fished from any bed is not always easy. A conclusion should be based on a sanitary survey of the waters. This should consider the area of the local waters, tides, currents and channels, etc. The nearness or remoteness of fecal pollution and its amount should also be determined. The bacterial examination of the waters and of the oysters at different points and at different tidal times as well as seasons, should also be employed as part of the sanitary survey and with special reference to flow, self-purification and dilution. The incidence of typhoid fever in the area may also influence the judgment. Re-inspections should be made.

The inshore oyster-house or shucking plants, the water supply for drinking or wet storage, the fishing-boats, personnel and facilities for caring for excretions must also be investigated as well as refrigeration for shucked stock.

We commented above on the fact that chlorination can at best result only in a diminution, not a complete cleansing of contaminated oysters. This does not imply that chlorination is valueless. Because of the greater dangers of inshore contamination, chlorination has distinct value in that it gives an expedient means of rendering safe the water used for drinking to condition oysters or for wet storage.

The licensing of oyster-beds should carry with it such regulations as will make it possible to trace oysters from the consumer to the source. A system of tagging, with retagging by intermedial jobbers, retention of tags with dates of sales in restaurants, oyster-bars, and fish-stores for one month are highly desirable--similar regulations should be enforced in the sale of clams for raw consumption.

Standard Methods for the Examination of Shellfish Adopted by the American Public Health Association. Oysters in the Shell.—*Selection of Sample.*—Twelve oysters of the average sizes of the lot under examination, with deep bowls, short lips and shells tightly closed, shall be picked out by hand and prepared for transportation to the laboratory. As complete a record of such data as is possible to obtain shall be made covering the following points: The exact location of the bed from which the sample has been selected. The depth of the water over the bed at time of collection. The state of the tide. The direction and velocity of the wind. Other weather conditions. The day and hour of the removal of the stock from the water. The conditions under which the stock has been kept since removal from the water and prior to the taking of the sample. The day and the hour of the taking of the sample.

Transportation of the Sample.—The oysters so selected shall be packed in suitable metal or pasteboard containers of such size and shape that a number of them can be enclosed in a shipping case capable of satisfactory refrigeration by means of ice. The important points in this connection are:

A. The prevention of the mixing of the oyster liquor of different samples, and of the mixing of the ice-water with the oysters.

B. The icing of the samples if they are not to arrive at the point of laboratory examinations inside of thirty-six hours or if the outside temperature is above 50° F.

It is not necessary to enclose the oysters in an absolutely tight container providing the above conditions are maintained.

Condition of Samples.—Record shall be made of the general condition of the oysters when received, especially whether the shells are open or closed; of the presence of abnormal odors and of the temperature of the stock.

Technical Procedure.—The bacteriological examination shall be started as soon as possible after the receipt of the sample.

The oysters shall be thoroughly cleaned with a stiff brush and clear running water and then dried. The edges of the shell shall be passed through the flame or burned with alcohol.

The opening of the shell shall be accomplished by either of the following methods:

A. By the use of a sterile oyster knife in the usual manner.

B. By drilling through a flamed portion of the shell near the hinge with a sterile drill. The drill shall be sterilized and the site of the operation in the shell be flamed at least once during the drilling process.

A satisfactory method of opening an oyster is to strike it a sharp blow with a hammer directly on the large muscle which holds the shells together. This injures the oyster just enough to cause the shells to spring apart. The edges of the shell are passed through the flame and the liquor is poured into a sterile test-tube.

Determination of Bacteria of the Colon-aërogenes Group.—Tests shall be made on a composite sample of each lot, obtained by pooling the shell liquor of at least five oysters. When less than five oysters are available the fact must be specifically stated. The quantitative determination of the presence of B. coli shall be in accordance with the following procedure:

Water used for dilution purposes shall be either sterile sea water or sterile tap water containing 2 per cent. sodium chloride.

Five 1-c.c. portions of the composite sample of shell liquor, 5 1-c.c. portions of 0.1 and 0.01 dilutions of the composite liquor shall be placed in fermentation tubes containing standard lactose broth. The fermentation tubes shall be incubated for two days at 37° C. Upon the formation of gas confirmatory tests shall be made in accordance with the standard methods of water analysis.

Expression of Results.—The results of the bacteriological examination for members of the colon-aërogenes group[1] shall be expressed by the following arbitrary numerical system, known as the American Public Health Association Method of Scoring Oysters.

If desired, the higher dilutions may be run for shucked oysters and for shell stock. In this case a 0.001 dilution has a positive value of 1000 and a 0.0001 dilution of 10,000 and so on.

The presence of B. coli in each fermentation tube, if confirmed, is to be given the following values, which represent the reciprocals of the greatest dilutions in which the test for B. coli is positive:

If present in 1 c.c., but not in 0.1 c.c., the value of 1.

If present in 0.1 c.c., but not in 0.01 c.c., the value of 10.

If present in 0.01 c.c. the value of 100.

The addition of these values for the five fermentation tubes gives the total value for the same and this figure is the score, the positive tube representing the greatest dilutions for each set being counted.

Results of Tests for B. Coli in Dilutions Indicated.

Oysters.	1 c.c.	0.1 c.c.	0.01 c.c.	Numerical value.
1	+	+	0	10
2	+	+	0	10
3	+	0	0	1
4	+	0	0	1
5	+	0	0	1
			Total or score for B. coli	= 23

+ = Presence of B. coli group in fermentation tube test with lactose bile where subsequent isolation tests have confirmed the results of the presumptive test or other satisfactory test.

0 = Failure to demonstrate presence of B. coli group.

[1] When the term B. coli is used it refers in all cases to bacteria of the colon-aërogenes group and not to the specific prototype.

Sometimes results similar to the following are obtained, that is, one or more oysters may show positive results in small quantities of shell water while an equal number may show negative results in larger quantities. In this case the next lower numerical value should be given to the positive results in the high dilutions and such positive results should be considered as being transferred to a lower dilution giving negative results in another oyster. This is done in order to avoid the unnatural result that could follow from what is probably an unequal distribution of the bacteria in the shell water. This recession of numerical values, however, should not be carried beyond the point where the number of such recessions is greater than the number of instances where other tubes in the series failed to give positive B. coli results.

As an example of the method of obtaining the score for B. coli, the following illustration is given.

Results of B. Coli Tests in Dilutions Indicated.

Oysters.	1 c.c.	0.1 c.c.	0.01 c.c.	Numerical value.
1	+	+	0	10
2	+	+	0	10
3	+	+	0	10
4	+	0	0	10 (not 1)
5	+	+	+	10 (not 100)
				Score, 50

Examination of Oysters Removed from the Shell, or Shucked Stock.—The procedure specified for oysters in the shell shall be followed, but attention is called to the fact that higher dilutions than 100 c.c. are usually required. Where the sample of shucked stock includes the oyster bodies, the standard procedure requires that 200 c.c. of the sample shall be added to an equal amount of sterile 2 per cent. salt solution, the mixture thoroughly shaken and the watery fluid used for the determination of B. coli as detailed above.

As the oyster meats are diluted with an equal amount of saline solution the result shall be multiplied by two for the score.

Clams and Other Shellfish.—The methods of examining clams and shellfish other than oysters shall be those given above. Certain modifications are necessary in the method of handling the sample and the opening of the shells, etc.

Clams are more likely to lose water during transportation than oysters. It is therefore necessary to take greater precautions to separate different samples of clams from each other than in the case of oysters.

Examination of Water.—The Standard Methods described in the chapter on Water are employed. For expression of the B. coli examination results, the scoring method described above is commonly employed.

Other Shellfish.—Mussel poisoning occurs. Gastro-intestinal symptoms, and in some cases, paralysis develops. Death may result. Apparently this only occurs where mussels are subject to sewage pollution. The poisoning has been attributed to a "ptomaine" mytilotoxin. Lobsters, crabs and shrimps, unless improperly kept, are not important as regards disease production. The untoward results of their consumption are usually due to individual food idiosyncrasy.

Official Use of the Score.—A score over 50 is commonly adopted as a basis for exclusion from market. No final B. coli rating based on these results shall be used for official[1] approval or condemnation unless positive confirmatory tests for the presence of organisms of the B. coli group shall have been obtained from the tube of highest or next highest dilution in each case. It should be emphasized that a high score suggests danger but that a low score does not necessarily indicate safety. In our experimentally contaminated oysters, contaminated with typhoid feces, we have encountered very low scores, where B. typhosus was present. In one instance B. typhosus was isolated but B. coli was not.

[1] There seems to be no special object gained in confirming the presumptive tests in oysters showing low scores. A low score is approved anyway and confirmation will not raise the score.

CHAPTER L.

THE SOIL BACTERIA AND THEIR FUNCTIONS. BACTERIA IN INDUSTRIES.

SOIL BACTERIA.

THE bacteria in the soil belong to many varieties. Some varieties are only accidentally present, being due to the contamination of the earth with the bacteria contained in animal feces and other waste products. The majority, however, pass their life and reproduce themselves chiefly or wholly in the soil. Many of these varieties have most important functions to perform in continuing the earth's food supply. Without them plant food, and therefore animal food, would cease to exist. Some make available for plants the carbon, nitrogen, hydrogen, and other compounds locked up in the dead bodies of animals and plants. Others construct food for plants from the gases of the air and the inorganic elements of the earth which in their simpler forms are not available.

The bacteria, together with the other somewhat less important microscopic plants and animals, thus form a vital link in the earth's life cycle of plants and animals. The bacteria in the soil require for their activities food, moisture, and a proper temperature. Some soil bacteria grow at or below a freezing temperature. They may be present to the extent of many millions in a single gram of rich loam, while in an equal quantity of sand they may be almost absent.

The various species associated together in the soil flora influence each other. Thus anaërobic bacteria are enabled to grow because of associated aerobes using up the free oxygen, while other species make assimilable substances not usable by others.

Decomposition.—This process is to some extent carried out through the agency of moulds and other fungi but it is chiefly due to the activities of bacteria. When this process is carried on in the absence of oxygen it is incomplete, giving rise to substances with unpleasant odors, such as H_2S, NH_3 and CH_4. This is called putrefaction. When oxygen is freely accessible more complete decomposition occurs with such end-products as CO_2, N and H_2O. These two processes, putrefaction and complete decay, cannot be sharply separated, as the second usually follows the first. The varieties of organisms causing these changes are many. Some groups will be found chiefly in decaying vegetable substances, others in animal tissues. They include all morphological forms of bacteria as well as yeasts and higher fungi. These forms exist everywhere in nature, although in various degrees, so that every bit of dead organic matter is sure to be decomposed if only moisture and warmth are present. The *B. subtilis* and *B. proteus vulgaris* groups are well-

known laboratory bacteria that are commonly found among decomposing materials. *B. proteus* is described under Pathogenic Bacteria.

B. Subtilis or Hay Bacillus.

The type form has the following characteristics (Fig. 214, page 745):

Source and Habitat.—Hay, straw, soil, dust, milk, etc.

Morphology.—Short, thick rods with round ends, sometimes form threads; sometimes also chains of long rods, short rods and coccus forms. 0.8μ to 1.2μ broad, 1.3μ to 3μ long. Often united in strings and threads.

Staining Reaction.—Stains by Gram's method.

Capsule, Flagella, Motility.—Bacilli possess a thin capsule and many flagella which are long and numerous; short forms actively motile; threads immobile.

Spore Formation.—Oval spores formed in presence of air germinating at right angles to long diameter. Spores are set free in about twenty-four hours, size 1.2μ by 0.6μ; widely distributed in nature, dust, air, excreta, etc. (Plate III, Fig. 23).

Biology: Cultural Characters (Including Biochemical Features).—Bouillon.—Uniformly cloudy growth with marked pellicle, wrinkled and thick; copious spore formation.

Gelatin Plates and Tubes.—Saucer-like depressions; colonies have granular centers and folded margins. Surface growth in stab cultures is whitish-gray; colonies sink on liquefaction of medium; liquefaction progresses in a cylindrical form, and a thick white scum is formed.

Agar Plates and Tubes.—Small, irregular, grayish-white colonies; moist glistening growth along needle track in stab cultures.

The bacteria in taking certain atoms from the molecules utilized in their growth leave the other atoms to enter into new relations and form new compounds. The actual products will depend on the decaying substance, the variety of bacteria and the conditions present.

The Splitting up of Carbon Compounds.—The plants form starch, and from it cellulose, woods, fats, and sugar. These substances once formed cannot be utilized by other generations of plants. Some of these are transformed in the bodies of animals, but the largest percentage await the activities of microörganisms. The sugars and starches usually undergo an alcoholic fermentation excited by the yeasts and moulds with the production of alcohol and carbon dioxide or an acid fermentation excited by bacteria with the production of acids and frequently of carbon dioxide.

Cellulose, which is so resistant to decay, is attacked by certain varieties of bacteria which are abundant in the soil. They act both in the presence and absence of free oxygen. Moulds also act on cellulose. Carbon dioxide, marsh gas, and other products are produced. Wood is apparently first attacked by the fungi and only later by other microörganisms. These bacteria are carried into the intestines and act upon cellulose and other substances.

The Decomposition of Nitrogenous Compounds.—Plants obtain their nitrogen chiefly in the form of nitrates. The small amount of usable nitrogen in the soil must be constantly replenished. This must come either from the nitrogen forming a part of protein materials or from the free nitrogen in the air.

Animals utilize the plant proteins and reduce them to much simpler compounds, such as urea, but even these are not suitable for plant use. We now know that microörganisms are employed to break com-

plex substances into simpler compounds and also to utilize the nitrogen of the air.

Denitrification.—This is a reducing process. The nitrate is made to yield up a part or all of its oxygen and thus becomes changed to nitrites and to ammonia and even to free nitrogen. The partial change does not rob the soil of its available nitrogen as does the total change, for the nitrites and ammonia may be changed by the nitrifying bacteria to nitrates. The dentrifying bacteria exist normally in most soils and are especially abundant in manure. Several well-known genera are especially active in the process of complete dentrification, for example, *B. coli, B. pyocyaneus, B. fluorescens.* There are three different types of nitrogen reduction: (1) The reduction of nitrates to nitrites and ammonia. (2) The reduction of nitrates and nitrites to gaseous oxides of nitrogen. (3) The reduction of nitrites with the development of free nitrogen gas.

Nitrification.—This is a process of oxidation by which through bacterial activities ammonia compounds are changed to nitrates and thus rendered utilizable by plants. This change is accomplished in two stages: first, the ammonia is oxidized to nitrite and, second, to nitrate. The nitrates are taken up by the plant roots from the soil. The bacterial nature of these changes were discovered in 1877 by two French investigators, Schloesing and Müntz. They noted that fermenting sewage after a time lost its ammonia and gained in nitrates, but that if the sewage was treated with antiseptics, so that fermentation ceased, no such change occurred. Warrington first and Winogradsky later more thoroughly investigated the bacterial cause of these changes. The latter by means of silica jelly, which contained no organic matter, was able to isolate two kinds of microbes, one in Europe and the other in America, which were able to change ammonia to nitrites. He called the one *nitrosomonas* and the other *nitrosococcus*. They are capable of acting on almost any ammonium salt. One variety of organisms capable of changing nitrites to nitrates was isolated, and this bacillus he called *nitrobacter*. These are small, slightly elongated bacilli. These bacteria are remarkable in that in pure cultures very small amounts of organic matter in the media act as antiseptics. They appear to be able to depend on mineral substances for their food. These bacteria are extremely important, for the plants take up most of their nitrogen in the form of nitrates. These changes are mostly produced in the surface soil. If the reaction of the soil becomes acid growth ceases. Soil bacteriologists are studying the nitrifying power of different types of soil under identical conditions. The process being one of oxidation the access of air is necessary.

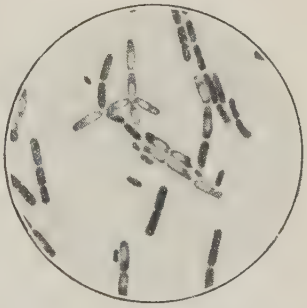


FIG. 214.—Bacillus subtilis with spores. Agar culture. Stained with gentian violet. The spores are unstained. × 1000 diameters. (Fränke.)

Nitrogen-fixing Bacteria.—Hellriegel, in 1886, and Wilfarth, in 1888, demonstrated that certain leguminous plants were able to use the nitrogen of the air and this apparently through the aid of bacteria growing in enlargements (tubercles) on the roots. After many trials Beijerinck (1888) succeeded in growing in pure cultures the bacteria germ in these root nodules. He gave them the name radicicola. The generic name is Rhizobium, given by Frank, who grew another variety the following year.

According to Ball,[1] there is no reasonable doubt but that *Rh. radicicola* and its relatives can and usually do remain active for very long periods in soil devoid of leguminous vegetation. Furthermore, these bacteria diffuse at a very considerable rate through soils that are in proper condition; therefore, if a soil should be found lacking the organisms, it is illogical to attempt to introduce them artificially without having first made the soil fit for their development.

It has not been shown by anyone that increased powers of resistance to unfavorable conditions, possessed by certain varieties, are at all correlated with their enhanced "greed for nitrogen." Moreover, it is far from being proved that any one race or "physiological species" is really more virile than another. Greig-Smith[2] showed that as many as three races are sometimes present in one and the same tubercle. Possibly, therefore, fixation of nitrogen may occur most rapidly only when two or more of these races are growing together.

In 1893 Winogradsky furnished proof that there are bacteria in the soil outside of the plant roots performing the same fnction as those within the roots. The first one he found doing this is the large anaërobic spore bearer, *Clostridium pasteurianum*. Then in 1901 Beijerinck found a group of aërobic bacteria that possess the same characteristic to a marked degree. He gave them the generic name Azobacter, describing two species. Lipman soon after identified several other species in this genus.

Beijerinck, in 1901, described two aërobic species of nitrogen-fixing bacteria. Later Bailey,[3] described three additional species. These were called *Azotobacter*. These studies have already led to the inoculation of soils and to the investigation of the kind of soils and crops best fitted for the growth of these bacteria. Many impoverished soils have already been greatly improved. There are probably many other varieties of bacteria capable of fixing nitrogen, because one can hardly examine the roots of any leguminous plants without finding different kinds of tubercles. The use of seed inoculated with the special variety of bacteria suitable for the plant and the soil is already largely practised.

Bacteria and Soil Minerals.—Some of the bacterial products act upon the inorganic constituents of the soil. The carbon dioxide and the organic acids act upon compounds of lime and magnesia, practically

[1] Centralbl. f. Bakt., etc., 1909, Abt. II, 23, 47.
[2] Jour. Soc. Chem. Indust., 1907, No. 7.
[3] Bacteria in Relation to Country Life.

insoluble in water, to form more soluble substances. The same is true of the rock phosphates, the silicates, of potassium sulphates, etc.

Scientific farming is beginning to make use of the knowledge already acquired, and there is reason to hope that great practical advantages will follow from the investigation of the relation of bacteria to soil exhaustion and replenishment.

The effect of excessive bacterial development appears at times to be harmful to the soil. Each crop seems to favor the growth of certain varieties, and the exhaustion of the soil which follows the constant raising of the same crop is now suspected to be due in part at least to the continuance of a few restricted species of bacteria in the soil which fail to produce all the substances necessary for the nutrition of the special crop and vegetation suffers, or again the bacteria finally entirely dissipate substances already in the soil necessary to growth.

The application of manure not only adds food for plant life, but also countless numbers of bacteria which make the food more available. The greatest number of bacteria are contained a little below the surface of the soil, where they are protected from drying and sunlight and are in contact with oxygen and with the roots and other food of the superficial soil.

THE PRESERVATION OF FOODS AGAINST DECOMPOSITION BY MICROÖRGANISMS.

The preservation of foods against decomposition by bacteria, yeasts, moulds, and higher fungi is obtained by using processes which will prevent the growth of microörganisms. Drying, exposure to wood smoke with consequent absorption of creosote, the addition of salt and sugar, of acids such as vinegar, spices, germicides such as boracic acid, formaldehyde, all are familiar methods of making foods unsuitable for bacterial growth.

Instead of using food preserved by drying or chemicals, products may be kept at temperatures too low for bacterial growth. Cold storage of meats, eggs, vegetables, etc., is now common.

The sterilization of food substances by heat with protection from infection afterward is made use of extensively in the canning of meats, fruits and vegetables. Care must be taken that absolutely all bacteria are killed, for otherwise decomposition will finally occur. The products of ordinary decomposition are now known to be much less deleterious than they were formerly thought to be.

Bacterial Fermentation in Relation to Miscellaneous Products.—Pasteur, in 1857, explained the process of fermentation as due to the action of microörganisms. He demonstrated that the change of sugar into lactic acid occurred only when living bacilli were present. If the fluid was sterilized the fermentation ceased. He stated that "organic liquids do not alter until a living germ is introduced into them." When the action is direct we speak of an organized ferment; when it is indirect, that is, due to the cell product, we call it an unorganized soluble ferment

or enzyme. Similar enzymes are produced by the cells of the animal tissues, such as ptyalin, pepsin, and trypsin. Pasteur's work led to the conclusion that the different fermentations were due to different varieties of organisms. The major part of fermentation is due to yeast. Some important fermentations are due to bacteria and a few to the moulds.

Wines and Beers.—Alcoholic Fermentation.—If there is a development of the yeast cells in a solution of grape-sugar we have a fermentation of the sugar with a final development of alcohol and carbon dioxide. It is thus that beers and wines are developed. When the carbohydrate is in the form of starch this is first converted into sugar and then later into the final products. If the sugar is in the form of saccharose, it is first changed by the yeast ferments to glucose. In all these three forms of fermentation the sugar is changed into alcohol and carbonic acid. When the alcohol reaches about 13 per cent. it stops further fermentation. These yeasts comprise a number of distinct varieties, some of which are cultivated while others, called "wild yeasts," propagate themselves. The distillery, brewery, and wine industries each make use of special yeasts and special conditions. The rising of bread is one of the most common uses of fermentation by yeast. The yeast acts upon the sugar made by the diastase from the starch. The resulting CO_2 and alcohol create myriads of little bubbles in the dough.

Diseases of Beer and Wines.—Hansen, Pasteur, and others demonstrated that the spoiling of beers and wines was due to the development of varieties of bacteria and yeasts which produce different kinds of fermentation from that desired. These produce alterations in flavor, bitterness, acidity.

Vinegar Making.—Vinegar is made from some weak alcoholic solution by the union of alcohol with oxygen. This oxidation can be brought about by a purely chemical process. When vinegar is formed in the usual way bacteria are essential. The scum on the surface of the fermenting alcohol is a mass of microörganisms. The mother of vinegar was named mycoderma by Pearson. Kutzing showed that this was composed of living cells. Hansen proved these to be bacteria. We now know there are many varieties of bacilli capable of producing this fermentation. They are grouped under the generic name Acetobacter. Each variety has its own optimum temperature and differs in the amount of acid it produces. Most of these have the peculiarity of growing at high temperatures into long threads without any traces of division. At low temperatures they produce long threads with swollen centers. The usual vinegar is made by using the variety of bacilli prevalent in the surroundings, but the custom is growing of adding to the pasteurized alcoholic solution the special variety desired in pure culture.

Sauerkraut.—This is cabbage leaves shredded, slightly fermented, and prevented from decay by the lactic acid bacteria. At first both yeasts and bacteria increase together, but with the increase in acidity all growth ceases. Putrefaction is prevented by the same cause. The lactic acid bacteria are the same as those found in sour milk.

Ensilage.—The fermentation is believed to be due partly to enzymes in the corn tissues and partly to bacterial action. The first changes are due chiefly to the enzymes.

The Curing of Tobacco.—The curing of tobacco is apparently due partly to bacterial processes and partly to the action of leaf enzymes.

The Bacterial or Virus Diseases of Plants.—These are probably as serious and varied for plants as for animals. The pear blight, the wilt disease of melons, the brown rot of tomatoes, the black rot of cabbages, and the mosaic diseases are examples. These plant diseases can be communicated experimentally from plant to plant by means of the pathogenic pure cultures of bacteria or the virus filtrates just as readily as animal diseases by their specific agents.

CHAPTER LI.

THE DESTRUCTION OF BACTERIA BY CHEMICALS. PRACTICAL USE OF DISINFECTANTS.

MANY substances, when brought in contact with bacteria, combine with their cell substance and destroy the life of the bacteria. While in the vegetative stage bacteria are much more easily killed than when in the spore form, and their life processes are inhibited by substances less deleterious than those required to destroy them.

Bacteria, both in the vegetative and in the spore form, differ among themselves considerably in their resistance to the poisonous effects of chemicals. The reason for this we believe is connected with the structure and chemical nature of their cell substance.

Chemicals are more poisonous at temperatures suitable for the best growth of bacteria than at lower temperatures, and act more quickly upon bacteria when they are suspended in fluids singly than when in clumps, and in pure water rather than in solutions containing organic matter. The increased energy of disinfectants at higher temperatures indicates in itself that a true chemical reaction takes place. In estimating the extent of the destructive or inhibitive action of chemicals, the following degrees are usually distinguished:

1. The growth is not permanently interfered with, but the pathogenic and zymogenic functions of the organism are diminished—*attenuation*. This loss of function is usually quickly recovered.

2. The organisms are not able to multiply, but they are not destroyed —*antiseptic*[1] action. When transferred to a suitable culture fluid free of the disinfectant these bacteria are capable of reproduction.

3. The vegetative development of the organisms is destroyed, but not the spores—incomplete or complete sterilization or disinfection, according as to whether spores are present in the organisms exposed and as to whether these spores are capable of causing infection.

4. Vegetative and spore forms are destroyed. This is complete *sterilization* or *disinfection*.[2]

The methods employed for the determination of the germicidal action of chemical agents on bacteria are, briefly, as follows:

If it is desired to determine the minimum concentration of the chemical substance required to produce complete inhibition of growth we proceed

[1] The word *antiseptic* is now being considered synonymous with *disinfectant* by government laboratories.

[2] Disinfection strictly defined is the destruction of all organisms and their products which are capable of producing disease. Sterilization is the destruction of all saprophytic as well as parasitic bacteria. It is not necessary in most cases to require disinfectants to be capable of sterilizing infected materials containing spores, for there are but few varieties of pathogenic bacteria which produce spores.

thus: A 10 per cent. solution of the disinfectant is prepared and 1 c.c., 0.5 c.c., 0.3 c.c., 0.1 c.c., of this is added to 10 c.c. of liquefied gelatin, agar, or bouillon, or, more accurately 10 c.c. minus the amount of solution added, in so many tubes. The tubes then contain 1 per cent., 0.5 per cent., 0.3 per cent., and 0.1 per cent. of the disinfectant. The fluid medium in the tubes is then inoculated with a platinum loopful of the test bacterium. The melted agar and gelatin may be simply shaken and allowed to remain in the tubes, and watched for any growth which takes place, or the contents of the tubes may be poured into Petri dishes, where the development or lack of development of colonies and the number can be observed. If no growth occurs in any of the dilutions, still higher dilutions are tested. Bacteria that have been previously injured in any way will be inhibited by much weaker solutions of chemicals than will vigorous cells. The same test can be made with material containing only spores.

If it is desired to determine the degree of concentration required for the destruction of vegetative development, the organism to be used is cultivated in bouillon, and into each of a series of tubes is placed a definite amount of diluted culture from which all clumps of bacteria have been filtered; to these a definite amount of watery solution of different percentages of the disinfectant is added. At intervals of one, five, ten, fifteen, and thirty minutes, one hour, and so on a small platinum loopful of the mixture is taken from each tube and inoculated into 10 c.c. of fluid agar or gelatin, from which plate cultures are made. Whenever it is probable that the antiseptic power of the disinfectant appears to be much greater than the germicidal, it is necessary to inoculate a second series of tubes from the first so as to decrease still further the amount of antiseptic carried over. The results obtained are signified as follows: x per cent. of the disinfectant in watery solution and at y temperature kills the organism in twenty minutes, z per cent. at the same temperature kills in one minute, and so on. If extreme accuracy is desired and there is any doubt whether the trace of disinfectant carried over with the platinum loop may have rendered the gelatin unsuitable for growth, control cultures should be made by adding bacteria which have been somewhat enfeebled by slight contact with the disinfectant to fluid to which a similar trace of the disinfectant has been added. If the strength of the disinfectant is to be tested for different substances it must be tested in these substances or their equivalent, and not in water.

The disinfectant to be examined should always be dissolved in an inert fluid, such as water; if on account of its being insoluble in water it is necessary to use another solvent, control experiments may be required to determine its action on the organism. Sometimes, as in the case of corrosive sublimate, the chemical unites with the cell substance to form an unstable compound, which inhibits the growth of the organism for a time before destroying it. If this compound is not broken up in the media it probably will not be in the body. In some tests it is of interest to break up this union and note then whether the organism is alive or dead.

In the above determinations the absolute strength of the disinfectant required is considerably less when culture media poor in albumin are employed than when the opposite is the case Cholera spirilla grown in bouillon containing no peptone or only 0.5 per cent. of peptone are destroyed in half an hour by 0.1 per cent. of hydrochloric acid; grown in 2 per cent. peptone bouillon, their vitality is destroyed in the same time on the addition of 0.4 per cent. HCl. In any case, all the organisms to be tested should be treated in exactly the same way and the results accompanied by a statement of the conditions under which the tests were made. It is now the custom to state the power of a disinfectant in terms of comparison with pure carbolic acid. A substance which had the same destructive power in a 1 to 1000 solution as carbolic acid in a 1 to 100 solution would be rated as of a strength ten times that of carbolic acid.

The following table gives the methods used and the results obtained with bichloride of mercury and with carbolic acid when their disinfecting action upon bacteria was tested out in the presence of blood serum.

TEST FOR THE DIFFERENCE OF EFFECT OF BICHLORIDE OF MERCURY AND CARBOLIC ACID SOLUTION ON TYPHOID BACILLI IN SERUM AND IN BOUILLON.

Time.	1′	3′	5′	10′	20′	30′	45′	1 hr.	1½ hrs.	2 hrs.	Strength of solution.
A. 2.5 c.c. serum and 2.5 c.c. $HgCl_2$ sol. 1:1000 Typhoid broth culture	+	+	+	−	−	−	−	−	−	−	Equals 1:2000 bichloride.
B. 2.5 c.c. bouillon and 2.5 c.c. $HgCl_2$ sol. 1:1000 Typhoid broth culture	−	−	−	−	−	−	−	−	−	−	Same.
C. 2.5 c.c. serum and 2.5 c.c. Carbolic sol. 5% Typhoid broth culture	+	+	−	−	−	−	−	−	−	−	Equals 2½% carbolic acid.
D. 2.5 c.c. bouillon and 2.5 c.c. Carbolic sol. 5% Typhoid broth culture	+	−	−	−	−	−	−	−	−	−	Same.

− Indicates total destruction of bacteria with no growth in media.
+ Indicates lack of destruction of bacteria with growth in media.

THE STANDARDIZATION OF DISINFECTANTS.

Rideal and Walker were the first to urge a useful method for standardizing disinfectants and their procedure is still used extensively.

The Public Health Service made certain changes in this method and adopted it officially as the method to be used by the Federal Departments in their work. It is generally used in the United States for determining the phenol coefficient of disinfectants and is the present Hygienic Laboratory method.[1]

No single method can serve as a means of comparing the value in practice of disinfectants of greatly diverse composition and destined

[1] Jour. Roy. Soc. Inst., 1903, **24**, 424; Hyg. Lab. Bull., April, 1912, p. 82; Pub. Health Repts., 1921, **36**, 1559.

for a variety of applications. However, disinfectants which are chemically related to phenol, which are to be used against organisms reacting similarly to the manner in which the typhoid bacillus reacts and which are destructive within the time and temperature limits of this test, may be compared as to their disinfecting properties within these limitations by means of this test. The results may be useful in the selection of a potent product, in making comparisons of cost in terms of service rendered, and in checking successive batches of the same product.

This method was submitted to several different laboratories for trial before its adoption, and the results seem to justify the belief that the personal equation in the performance of the tests does not play an inordinate rôle.

The Test Culture.—The test culture is a culture of Bacillus typhosus, Hopkins strain. Between periods of testing it is maintained on nutrient agar stabs, transferred at monthly intervals.

For at least five days before the test the culture is transferred at twenty-four hour intervals to successive tubes of the meat extract broth described below and incubated at 37° C. Transfers are made with one standard loopful. The culture is filtered through sterile filter paper just before using. The test is performed with a twenty-four-hour culture.

The Phenol.—The phenol must comply with the requirements of the Eighth United States Pharmacopœia. Particularly the congealing point must not be below 40° C. The crystals are kept in tightly stoppered amber-colored bottles in a dark and relatively cool place.

A 5 per cent. original solution is made by adding 1 part by weight of phenol, liquefied by warming the bottle, to 19 parts of distilled water. A fresh solution is made for each day's use.

The Culture Medium.—Make meat extract medium as follows:

Beef extract (Liebig's)	3 gm.
Peptone (Armour's, for disinfectant testing)	10 "
Sodium chloride	5 "
Water, distilled	1000 c.c.

Boil for fifteen minutes.
Make up to original weight by addition of water.
Filter through paper.
Tube, 10 c.c. to each tube.
Sterilize.
The pH value of this medium should be between 6 and 7.

Glassware and Apparatus.—Glassware for measuring must be accurately graduated. It must be clean, dry, and sterile at the time of use. There will be needed:

1 c.c. capacity pipettes.
5 c.c. capacity pipettes.
1 c.c. delivery pipettes, graduated in tenths.
5 c.c. delivery pipettes.
100 c.c. measuring cylinders, graduated in 1 c.c., glass stoppered.
Seeding tubes, 1 x 3 inches, flared tops, round bottoms.
Racks consisting of blocks of wood with rows of holes for both the

seeding tubes (before they are placed in the water-bath) and the subculture tubes.

Wire loops must be carefully made and kept from damage. They are made as follows: A close cylindrical spiral is made by winding a piece of platinum wire, No. 23, B. and S. gauge, as tightly as possible about a piece of steel or other hard wire having a diameter of 0.072 inch (No. 13, B. and S. gauge) to complete a little more than four full turns. The long end of the wire is then bent sharply at right angles to the wound portion and parallel to the steel wire. The core is removed and the short end of the wire is clipped off so as to leave exactly four full turns to the coil. The successive turns of the spiral must touch one another continuously. The long end of the wire is attached to an aluminum handle.

A convenient support is provided on which to rest the loops so that a bat-wing Bunsen burner may be placed under each one successively.

A constant temperature bath is provided, capable of maintaining the seeding tubes at 20° C. during the time of the test. A well-insulated bath of large volume relative to the surface exposed is sufficient without thermo-regulating appliances.

Disinfectant Testing Machine.—The use of a disinfectant testing machine is optional. One is described in Reprint No. 462 from the *Public Health Reports*. A few modifications have proved useful. For example, the use of platinum instead of nichrome loops, and the practice of sterilizing the subculture tubes covered with padded inverted troughs in the racks.

Dilutions.—Dilutions of phenol and of disinfectants are made from the original liquid on the day of the test. For the dilutions of the disinfectant, a 5 per cent. solution is made by adding 5 c.c. of the disinfectant to 95 c.c. of sterile distilled water. A standardized 5 c.c. capacity pipette is used for this purpose. After filling the pipette, all excess of the disinfectant on the outside of the pipette is wiped off with sterile gauze. The contents of the pipette are then delivered into a cylinder containing 95 c.c. of sterile distilled water and the pipette is washed out as clean as possible by aspiration and blowing out the contents into the cylinder. The contents of the cylinder are then thoroughly shaken and the dilutions up to 1 to 500 are made from it, using delivery pipettes for measuring. For those disinfectants which do not readily form a 5 per cent. solution, make a 1 per cent. solution, and from this make the dilutions greater than 1 to 100. If greater dilutions than 1 to 500 are to be made, a 1 per cent. solution is made from the 5 per cent. solution and the higher dilutions are made from this.

For the higher dilutions, delivery pipettes may be used. The following scale is used for making dilutions:

For dilutions up to 1 to 70, increase or decrease by a difference of 5 (*i. e.*, 5 parts of water); from 1 to 70 to 1 to 160, by a difference of 10; from 1 to 160 to 1 to 200, by a difference of 20; from 1 to 200 to 1 to 400, by a difference of 25; from 1 to 400 to 1 to 900, by a difference of 50; from 1 to 900 to 1 to 1800, by a difference of 100; from 1 to 1800 to 1 to 3200, by a difference of 200; and so on if higher dilutions are necessary.

It is important that the cylinders used for making the dilutions be correctly graduated. It is preferable to use standardized cylinders and pipettes.

Method.—This description applies to the hand method. For the use of the machine, follow the procedure of Reprint No. 462 from the *Public Health Reports.*

The object is to add 0.1 c.c. of typhoid culture to 5 c.c. of successive dilutions of the disinfectant and of phenol, and, after these additions, to transfer a loopful of each mixture to a separate subculture tube at periods of five,[1] seven and a half, ten, twelve and a half and fifteen minutes. The subculture tubes are then incubated for forty-eight hours at 37° C., and readings of growth or no growth are made and recorded.

Dilutions are made to cover the expected range of the disinfectant, and 5 c.c. of each dilution is placed in a seeding tube. Dilutions of phenol are made as follows: 1 to 80, 1 to 90, 1 to 100, 1 to 110, 1 to 120 and 1 to 130, and 5 c.c. of each is placed in a seeding tube.

The seeding tubes are placed in the water-bath at 20° C. and a few minutes are allowed for their contents to reach this temperature.

To each seeding tube 0.1 c.c. of culture is then added seriatim, allowing fifteen seconds for each addition. If there are 10 tubes of disinfectant dilutions, this will occupy two and a half minutes. At the end of five minutes from the time of adding the disinfectant to the first seeding tube, a loopful of the mixture is transferred from this tube to a subculture tube, and this is done from each successive seeding tube at fifteen-second intervals. This procedure is repeated after the lapse of seven and a half, ten, twelve and a half and fifteen minutes from the time of the first addition of culture to the seeding tube. Each loop is placed on the support and flamed with the Bunsen burner immediately after use, and the use of several loops permits them to cool before they are needed again. The operator is therefore obliged to make a transfer every fifteen seconds for ten minutes.

The culture is best added to the seeding tube by holding the latter in a slanting position and touching the tip of the 1 c.c. pipette to the wetted surface exposed on its upper side. One-tenth c.c. is run in at the proper time and thorough admixture is assured by gentle shaking.

The dilutions of phenol are next treated in the same manner as those of the disinfectant.

The tubes are properly labeled and are placed in the incubator for forty-eight hours, at the end of which time readings of growth or no growth are made and entered in a table as + or − signs respectively.

In the nature of the test it is unavoidable that discrepancies in the even oblique slant of the plus signs across the chart will occasionally be encountered; but if these are numerous, a faulty technic is indicated and the test should be discarded. The same applies to accidentally contaminated tubes.

[1] A two and a half minute period may also be employed to show that the organisms survive.

Determination of Coefficient.—The coefficient is the arithmetic mean of the sum of three ratios. These ratios are, the denominator of the highest dilution of the disinfectant in whose subculture tube no growth occurs, divided by the corresponding figure for phenol, for the five, ten and fifteen minute intervals, respectively.

Sample.	Dilution.	Time of exposure in minutes. 5	7½	10	12½	15
Disinfectant . . .	1 to 700	−	−	−	−	−
	1 to 800	+	−	−	−	−
	1 to 900	+	+	−	−	−
	1 to 1000	+	+	+	+	−
	1 to 1100	+	+	+	+	+
Phenol	1 to 80	−	−	−	−	−
	1 to 90	+	−	−	−	−
	1 to 100	+	+	+	+	−
	1 to 110	+	+	+	+	−
	1 to 120	+	+	+	+	+
	1 to 130	+	+	+	+	+

$$\text{Coefficient} = \frac{\frac{700}{80} + \frac{900}{90} + \frac{1000}{110}}{3} = \frac{8.7 + 10.0 + 9.0}{3} = 9.2$$

All Disinfectants vary and some widely in their germicidal properties, depending upon the presence or absence of organic matter. As under practical conditions organic matter is usually present, it is of some importance to know how far it decreases the efficiency of disinfection.

For the purpose of obtaining comparable results, Anderson and McClintic have suggested the use of peptone 10 per cent. and gelatin 5 per cent. in distilled water. One part of the culture is mixed with 10 parts of the organic solution, 1.1 c.c. then being added to a series of dilution tubes containing 4 c.c. of diluted disinfectant. In determining the coefficient allowance must be made for the added amount of organic matter.

The modified methods of Anderson and McClintic[1] are called the "hygienic laboratory phenol coefficient," with or without organic matter. Any organic matter may be used in the test to approach the special conditions under which a disinfectant is to be used.

In comparing the value of disinfectants the cost as well as the coefficient must be considered. This is best stated in terms of the relative cost of 100 units of efficiency as compared with pure phenol = 100, thus:

$$\frac{\text{Cost of disinfectant per gallon}}{\text{Cost of phenol per gallon}} (= \text{Cost ratio}) \div \frac{\text{Coefficient of disinfectant}}{\text{Coefficient of phenol} = (1).}$$

$(= \text{the efficiency}), \times 100 = \text{relative cost per 100 units.}$

The factors to be considered in the selection of a disinfectant have been summed up by McCoy as follows: (1) Availability; (2) efficiency; (3)

[1] See Hygienic Laboratory Bulletin No. 82, for further details and apparatus for simplifying the steps of the test.

ease of application; (4) danger to man; (5) deleterious effect on materials; (6) length of exposure required, and (7) cost.[1]

The Food, Drug and Insecticide Administration of the Department of Agriculture has charge of regulatory work in matters pertaining to insecticides and fungicides, including disinfectants, passing interstate. Their attitude is that a disinfectant should be tested not only against B. typhosus with the object of obtaining the phenol coefficient but that the action against Staphylococcus aureus, B. diphtheriæ, B. tuberculosis, Diplococcus pneumoniæ, Streptococcus hemolyticus and B. pestis should also be studied where the preparation is recommended for general use. These organisms represent the suppurative group, granular Gram-positive group and diphtheroids, acid-fast group, encapsulated Gram-positive cocci, septicemia pathogens and hemorrhagic-septicemic group. When the chemical is to be used on animals they include tests on Staphylococcus aureus, Streptococcus equine, B. abortus Bang, Salmonella enteritidis, B. pollurum (fowls) and B. sanguinarium. They do not recommend the abandonment of the usual phenol coefficient test which they believe is useful for manufacturing control, but believe that labels should state the percentage strength to be used for general disinfection, which shall have been determined by testing against the above organisms.[2]

The Bureau of Animal Industry of the Department of Agriculture is charged with disinfection of premises and vehicles which may have become infected by diseased animals in the course of interstate transportation as well as the animals themselves and promulgates chemical formulas for permitted disinfectants.

Antiseptic Value.—In some cases there is sufficient disinfectant carried over by the loop to exert antiseptic action, and growth does not occur. If this is not taken into consideration a disinfectant will be given an excessively high coefficient. No satisfactory method has been devised to avoid this difficulty. The inoculated broth tube may be shaken and a loop or more inoculated from it to second broth tube, in this way diluting the disinfectant still further.

Chick[3] has attempted to overcome the difficulty in the case of mercury-containing disinfectants by adding to each tube of broth 0.2 c.c. of a saturated watery solution of hydrogen sulphide.

Many substances which are strong disinfectants become altered by the conditions under which they are used, so that they lose a portion or all of their germicidal properties; thus, quicklime and milk of lime act by means of their alkali and are disinfecting agents only so long as sufficient calcium hydroxide is present. If this is changed by the carbon dioxide of the air into carbonate of lime it becomes harmless. Bichloride of mercury and many other chemicals combine with various organic and inorganic substances to form compounds which, though still germicidal, are much less so than the original substances. Solutions of chlo-

[1] Disinfectants—Public Health Bulletin No. 42, 1917.

[2] Reddish: The Newer Knowledge of Bacteriology and Immunity, Jordan and Falk, 1928, p. 301.

[3] Journal of Hygiene, 1908, **8**, 654.

rine, peroxides, etc., when in contact with an excess of organic matter soon become inert because of the chemical compounds formed.

The Disinfecting Properties of Inorganic Compounds.—Bichloride of Mercury.—This substance, soluble in 16 parts of cold water, will kill some varieties of bacteria in a few minutes when present in water in 1 to 50,000 dilution, while others, such as tubercle bacilli resist much stronger solutions, and inhibits the growth of most forms of bacteria when present 1 to 100,000 in a medium of nutrient bouillon. With organic substances its power is lessened, so that 1 part to 1000 may be required. Most spores are killed in 1 to 500 watery solution within one hour. Corrosive sublimate is more effective as a germicide in watery solution than it is in alkaline fluids containing much albuminous substance. In such fluids, besides loss in other ways, precipitates of albuminate of mercury are formed which are at first insoluble, so that a part of the mercuric salt does not really exert any action. In fluids such as blood, blood serum, pus, sputum, tissue fluids, etc., the soluble compounds of mercury are largely converted into insoluble compounds.

For ordinary use, where corrosive sublimate is employed, solutions of 1 to 500 and 1 to 2000 will kill the vegetative forms of bacteria in from one to twenty minutes. The stronger solution to be used when much organic matter is present.

Mercuric chloride volatilizes slowly and it is better to wash off walls after use of bichloride solutions. Solutions of this salt should not be kept in metal receptacles. Mercuric chloride solution has disadvantages in that it corrodes metals, irritates the skin, and forms almost inert compounds with albuminous matter. In order to avoid accidents, solutions of this odorless disinfectant should be colored by some dye.

Biniodide of Mercury.—This salt is very similar in its effect to the bichloride.

Nitrate of Silver.—Nitrate of silver in watery solution has about one-fourth the value of the bichloride of mercury as a disinfectant, but nearly the same value in inhibiting growth. In albuminous solutions it is equal to bichloride of mercury. Compounds of silver nitrate and albuminous substances such as argyrol, protargol and neosilvol, which are otherwise insoluble and are kept in solution by adding a protective colloid, have been used because of the absence of irritative properties combined with moderate antiseptic power.

Sulphate of Copper.—This salt has about 50 per cent. of the value of mercuric chloride. It has a remarkable affinity for many species of algæ, which are destroyed in water containing 1 part per million. Typhoid bacilli are destroyed in twenty-four hours by a dilution of 1 to 400,000 when the water has no excessive amount of organic material. It is not known to be poisonous in this strength, so that it can be added to water supplies temporarily.

Sulphate of Iron.—This is a much less powerful disinfectant than sulphate of copper. A 5 per cent. solution requires several days to kill the typhoid bacilli. It can be considered only as a mild antiseptic and deodorant.

Zinc Chloride.—This is very soluble in water, but is a much weaker disinfectant than copper sulphate.

Sodium Compounds.—A 30 per cent. solution of NaOH kills anthrax spores in about ten minutes, and a 4 per cent. solution in about forty-five minutes. One per cent. kills almost all vegetative forms in a few minutes. Sodium carbonate kills spores with difficulty even in concentrated solution. Together with heat as great as 85° C. spores are killed in ten minutes. It is used frequently to cover metallic instruments. A 5 per cent. solution kills in a short time the vegetative forms of bacteria. Even ordinary soapsuds have a slight bactericidal as well as a marked cleansing effect. The bicarbonate has almost no destructive effect on bacteria. B. tuberculosis is very resistant to alkalies.

Calcium Compounds.—Calcium hydroxide, $Ca(OH)_2$, is a powerful disinfectant; the carbonate, on the other hand, is almost without effect. The former is prepared by adding one pint of water to two pounds of lime (quicklime, CaO). Exposed to the air the calcium hydrate slowly becomes the inert carbonate. A 1 per cent. watery solution of the hydroxide kills bacteria which are not in the spore form within a few hours. A 3 per cent. solution kills typhoid bacilli in one hour. A 20 per cent. solution well mixed with equal parts of feces or other filth will effect complete sterilization within two hours.

Effects of Acids.—An amount of acid per liter which is equivalent to 40 c.c. of normal hydrochloric acid is sufficient to prevent the growth of all varieties of bacteria and to kill many. Twice this amount destroys most bacteria within a short time. The particular kind of acid makes little difference. Bulk for bulk, the mineral acids are more germicidal than the vegetable acids, but that is because their molecular weight is so much less. A 1 to 500 solution of sulphuric acid kills typhoid bacilli within one hour. A similar solution of hydrochloric acid is about one-third weaker, and acetic acid somewhat weaker still. Citric, tartaric, malic, formic and salicylic acids are similar to acetic acid. Boric acid in 2 per cent. solution destroys the less resistant bacteria and inhibits the others.

Gaseous Disinfectants.—The germicidal action of gases is much more active in the presence of moisture than where conditions are dry.

Sulphur Dioxide (SO_2).—Numerous experiments have been made with this gas owing to the fact that it has been so extensively used for the disinfection of hospitals, ships, apartments, clothing, etc. This gas is a much more active germicide in a moist than in a dry condition, due, no doubt, to the formation of the more active disinfecting agent—sulphurous acid (H_2SO_3). In a pure state anhydrous sulphur dioxide does not destroy spores and is not certain to destroy bacteria in the vegetative form. Sternberg has shown that the spores of *Bacillus anthracis* and *Bacillus subtilis* are not killed by protracted contact with liquid SO_2 (liquefied by pressure). Koch found that various species of spore-bearing bacilli were not destroyed by a ninety-six hour exposure in a disinfecting chamber to the action of SO_2 in the proportion of 4 to 6 per cent. by volume. In the absence of spores, however, the

anthrax bacillus in a moist condition, attached to silk threads, was found by Sternberg to be destroyed in thirty minutes in an atmosphere containing 1 per cent. by volume.

As the result of a large number of experiments with SO_2 as a disinfectant it has been determined that an "exposure for eight hours to an atmosphere containing at least 4 per cent. by volume of this gas *in the presence of moisture*" will destroy most, if not all, of the pathogenic bacteria in the absence of spores. Four pounds of sulphur burned for each 1000 cubic feet will give an excess of gas.

At present SO_2 in disinfection is used only for the purpose of killing vermin.

Peroxide of Hydrogen (H_2O_2).—This is an energetic disinfectant when the bacteria are suspended in fluids, such as water, in which there is but little organic matter; in 2 per cent. solution (about 40 per cent. of the ordinary commercial article) it will kill the spores of anthrax in two to three hours. A 20 per cent. solution of a good commercial hydrogen peroxide solution will destroy quickly the pyogenic cocci and other spore-free bacteria. It combines with organic matter, becoming inert. It is prompt in its action and not poisonous, but apt to deteriorate if not properly kept.

Chlorine.—Chlorine is a powerful gaseous germicide, owing its activity, it has been claimed, to the formation of HOCl (hypochlorous acid) and the subsequent release of nascent oxygen when it comes in contact with microörganisms in moist condition. It is, therefore, a much more active germicide in the presence of moisture than in a dry condition. Thus, Fischer and Proskauer found that dried anthrax exposed for an hour in an atmosphere containing 44.7 per cent. of dry chlorine were not destroyed; but that spores previously moistened and exposed in a moist atmosphere for the same length of time were destroyed by 4 per cent. chlorine, and even 1 per cent. was effective when the time was extended to three hours. The anthrax bacillus, in the absence of spores, was killed by a twenty-four-hour exposure to 1 part of chlorine in 2500 of moist air. In watery solutions 0.2 per cent. kills spores within five minutes and the vegetative forms almost immediately. In water containing little organic matter, 1 part per million destroys typhoid bacilli within a few hours. In water with more organic matter a proportionately greater amount is required.

Chlorinated Lime (Called "Chloride of Lime").—Chlorinated lime is made by passing nascent chlorine gas over unslaked lime. It should not contain less than 10 per cent. of available chlorine, and can now be obtained containing 30 per cent. It should have a strong odor of chlorine. Its efficiency depends upon the chlorine it contains in the form of hypochlorites. The calcium hypochlorite is readily broken up into hypochlorous acid. A solution of 0.5 to 1 per cent. of chlorinated lime in water will kill most bacteria in one to five minutes. and 1 part in 100,000 will destroy typhoid bacilli in twenty-four hours. Spores are usually destroyed within one hour by a 5 per cent. solution. Chlorinated lime not only bleaches but destroys fabrics.

The Hypochlorites (Labarraque's Solution Containing Chiefly Sodium Hypochlorite).—Solutions of hypochlorites are practically the same as solutions of chlorinated lime and are much more expensive.

Bromine and Iodine, in the moist condition, are about as valuable as chlorine for gaseous disinfection but, owing to their poisonous and destructive properties, they, like chlorine, are not suitable for general use in house disinfection; they have a use, however, in sewers and similar places.

Trichloride of Iodine in 0.5 per cent. solution destroys the vegetative forms of bacteria in five minutes.

Organic Disinfectants.—Alcohol in 10 per cent. solution inhibits the growth of bacteria; absolute alcohol kills bacteria in the vegetative form in from several to twenty-four hours. According to Epstein, 50 per cent. alcohol (in water) has more germicidal power than any other strength, the power gradually diminishing with both stronger and weaker solutions.

Formaldehyde.—Formaldehyde, or formic aldehyde, was isolated in 1867 by von Hoffmann, who obtained it by passing the vapors of methyl alcohol mixed with air over finely divided platinum heated to redness. The methyl alcohol is oxidized and produces formaldehyde as follows:

$$CH_3OH + O = CH_2O + H_2O$$

Formaldehyde is a gaseous compound possessed of an extremely irritating odor. At a temperature of 68° F. the gas is polymerized—that is to say, a second body is formed by a union of two molecules of CH_2O. This is known as paraformaldehyde, and is a white, soapy body, soluble in boiling water and in alcohol. Formaldehyde is sold commercially as a clear, watery liquid containing from 33 to 40 per cent. of the gas and 10 to 20 per cent. of methyl alcohol, its chief impurity. If the commercial solution—ordinarily known in the trade as "formalin"—is evaporated or concentrated above 40 per cent., paraformaldehyde results; and when this is dried *in vacuo* over sulphuric acid a third body—trioxymethylene—is produced, consisting of three molecules of CH_2O. This is a white powder, almost insoluble in water or alcohol, and giving off a strong odor of formadelyde. The solid polymers of formaldehyde, when heated, are again reduced to an active gaseous condition; ignited, they finally take fire and burn with a blue flame, leaving but little ash. When burned they have no germicidal properties.

Formaldehyde has an active affinity for many organic substances, and forms with some of them definite chemical combinations. It combines readily with ammonia to produce a compound called hexamethylene tetramine, which possesses neither the odor nor the antiseptic properties of formaldehyde. This action is made use of in neutralizing the odor of formaldehyde when it is desired to dispel it rapidly after disinfection.

Formaldehyde also forms combinations with certain anilin colors—viz. fuchsin and safranin—the shades of which are thereby changed or intensified. These dyes are used in tests for aldehydes. They are the only colors, however, which are thus affected, and as they are seldom used in dyeing, owing to their liability to fade, this effect is of little practical significance. The most delicate fabrics of silk, wool, cotton, fur, etc., are unaffected in texture or color by formaldehyde. Iron and steel are attacked, after long exposure, by the gas in combination with watery vapor; but copper, brass, nickel, zinc, silver and gilt work are not acted upon at all.

Formaldehyde unites with nitrogenous products of decay—fermentation or decomposition—forming true chemical compounds, which are odorless and sterile. It is thus a true deodorizer in that it does not replace one odor by another more powerful, but forms new chemical compounds which are odorless. Formaldehyde has a peculiar action upon albumin, which it transforms into an insoluble and indecomposable substance. It renders gelatin insoluble in boiling water and in most acids and alkalies. It is from the property of combining chemically with the albuminoids forming the protoplasm of bacteria that formaldehyde is supposed to derive its bactericidal powers. Formaldehyde is an excellent preservative of organic products. It has been used for the preservation of meat, milk, and other food products; but, according to Trillat and other investigators, formaldehyde renders these substances indigestible and unfit for food. It is successfully employed as a preservative of pathological and histological specimens.

There are on record no exact experiments dealing with the physiological action on the human subject of formaldehyde taken internally. A considerable quantity of 1 per cent. solution has been taken without serious results; and trioxymethylene has been given as an intestinal antiseptic in doses up to 90 grains. According to Aronson rabbits and guinea-pigs allowed to remain for twelve and twenty-four hours in rooms which were being disinfected with formaldehyde gas were found to be apparently well when the rooms were opened. On autopsy the animals showed no injurious effects of the gas. Others have noted that occasionally animals, such as dogs and cats, which have accidentally been confined for any length of time in rooms undergoing formaldehyde disinfection died from the effects of the gas. Many observers, however, have reported that insects, such as roaches, flies, and bed-bugs are not, as a rule, affected. These observations would seem to indicate that although formaldehyde is comparatively non-toxic to the higher forms of animal life, nevertheless a certain degree of caution should be observed in its use. It is important to remember that formaldehyde in gaseous form is practically inert as an insecticide except in great concentration.

The researches of Pottevin and Trillat have shown that the germicidal power of the gas depends not only upon its concentration, but also upon the temperature and the condition of the objects to be sterilized. As with other gaseous disinfectants—viz., sulphur dioxide and chlorine—it has been found that the action is more rapid and complete at high temperatures—*i. e.*, at 35° to 45° C. (95 ° to 113° F.)—and when the test objects are moist, than at lower temperatures and when the objects are dry. Still, it has been repeatedly demonstrated by

actual experiment that it is possible by an exposure of a few hours to a saturated atmosphere of formaldehyde gas, to disinfect the surface of apartments and articles contained in them, under the conditions of temperature and moisture ordinarily existing in rooms even in winter. The results of numerous experiments have shown that the presence in the air of 2.5 per cent. by volume of the aqueous solution, or 1 per cent. by volume of the gas, are sufficient to destroy within a few minutes fresh virulent cultures of the common non-spore-bearing pathogenic bacteria.

Stahl has shown that bandages and iodoform gauze can be kept well sterilized by placing in the jars tablets of paraformaldehyde containing 50 per cent. of formaldehyde. The same experimenter has also succeeded in making carpets and articles of clothing germ-free by spraying them with 0.5 to 2 per cent. solution of formaldehyde for fifteen to twenty-minutes without affecting in any way the color of the fabrics. The investigations of Trillat, Aronson, Pottevin, and others have shown that a concentration of $\frac{1}{10000}$ of the 40 per cent. aqueous solution (equal to $\frac{1}{25000}$ of pure formaldehyde) was safe and sufficiently powerful to retard bacterial growth.

In our experiments formalin has upon the vegetative forms of bacteria about one-half the destructive power of pure carbolic, a 2 per cent. watery solution of formalin being effective within five to thirty minutes.

Chloroform ($CHCl_3$).—This substance, even in pure form, does not destroy spores, although a 1 per cent. solution kills bacteria in vegetative form. Chloroform is used practically as an antiseptic in antitoxic sera and in blood serum for culture purposes. The chloroform is expelled from the serum by heating it to 55° C.

Iodoform (CHI_3).—This substance has very little destructive action upon bacteria; indeed, upon most varieties it has no appreciable effect whatever. When mixed with putrefying matter, wound discharges, etc., the iodoform is reduced to soluble iodine compounds, which act partly by destroying the bacteria and partly by uniting with poisons already produced.

Carbolic Acid (C_6H_5OH).—Pure phenol crystallizes in long, colorless crystals. In contact with air it deliquesces. It has a penetrating odor, a burning taste, and is a corrosive poison. It is soluble at ordinary temperatures in about 15 parts of water but dissolves with some difficulty and, therefore, should be thoroughly mixed. It is not destructive to fabrics, colors, metals, or wood, and does not combine as actively with albuminous matters as does bichloride of mercury. It is therefore more suitable for the disinfection of feces, etc. A solution of 1 part to 1000 inhibits the growth of bacteria; 1 part to 400 kills the less resistant bacteria, and 1 part to 100 kills the remainder. A 5 per cent. solution kills the less resistant spores within a few hours and the more resistant in from one day to four weeks. A slight increase in temperature aids the destructive action; at 37.5° C. spores are killed in three hours. A 3 per cent. solution kills streptococci, staphylococci, etc., within one minute. Carbolic acid loses much of its value when in solution in alcohol or ether and carbolic acid burns should be immediately neu-

tralized with alcohol. An addition of 0.5 HCl aids its activity. Carbolic acid is so permanent and so comparatively little influenced by albumin that it is widely used in practical disinfection even in place of more powerful substances.

Cresol.—Cresol [$C_6H_4(CH_3)OH$] is the chief ingredient of the so-called "crude carbolic acid." This is almost insoluble in water, and therefore of restricted value. Many methods are used for bringing it into solution so as to make use of its powerful disinfecting properties. With equal parts of crude sulphuric acid it is a powerful disinfectant, but it is, of course, strongly corrosive. Creolin is one of many disinfectants prepared by dissolving crude carbolic acid in soaps, principally rosin soap. It is used in 1 to 5 per cent. emulsions. It is fully as powerful as pure carbolic acid. There are several varieties under this name, with varying compositions. They contain phenol, cresols, hydrocarbons and soap. Lysol consists largely of pure cresols and vegetable oil soaps and gives clear solutions in water instead of emulsions.

Tricresol.—Tricresol is a refined mixture of the three cresols (metacresol, paracresol, and orthocresol). It is soluble in water to the extent of 2.5 per cent. and its disinfecting power is about three times as great as that of carbolic acid.

Oil of Turpentine, 1 to 200, prevents the growth of bacteria.

Camphor has very slight antiseptic action.

Creosote 1 to 200 kills many bacteria in ten minutes; 1 to 100, however, failed to kill tubercle bacilli in twelve hours.

Essential Oils.—Caldéac and Mennier[1] found that the essences of cinnamon, cloves, thyme, and other essential oils killed typhoid bacilli within one hour. Sandalwood required twelve hours.

Thymol and eucalyptol have about one-fourth the strength of carbolic acid (Behring).

Oil of peppermint in 1 to 100 solution prevents the growth of bacteria.

TABLE OF ANTISEPTIC VALUES.[2]

Alum	1 to 222	Mercuric chloride	1 to 14,300
Aluminum acetate	1 to 6000	Mercuric iodide	1 to 40,000
Ammonium chloride	1 to 9	Potassium bromide	1 to 10
Boric acid	1 to 143	Potassium iodide	1 to 10
Calcium chloride	1 to 25	Potassium permanganate	1 to 300
Calcium hypochlorite	1 to 1000	Pure formaldehyde	1 to 25,000
Carbolic acid	1 to 333	Quinine sulphate	1 to 800
Chloral hydrate	1 to 107	Silver nitrate	1 to 12,500
Cupric sulphate	1 to 2000	Sodium borate	1 to 14
Ferrous sulphate	1 to 200	Sodium chloride	1 to 6
Formaldehyde (40 per cent.)	1 to 10,000	Zinc chloride	1 to 500
Hydrogen peroxide	1 to 20,000	Zinc sulphate	1 to 20

[1] Ann. de l'Inst. Past., 1889, **3**, 317.

[2] These figures are approximately correct, and represent the minimum percentage of "disinfectant" to be added to a fluid containing considerable organic material, in order permanently to prevent any bacterial growth. Solutions of half the given strength will inhibit the growth of most bacteria and prevent the growth of many varieties.

See Disinfectants, Their Use and Application in the Prevention of Communicable Diseases, McCoy, *et al.*, Public Health Bulletin No. 42, 1917.

CHAPTER LII.

PRACTICAL DISINFECTION AND STERILIZATION (HOUSE, PERSON, INSTRUMENTS AND FOOD). STERILIZATION OF MILK FOR INFANT-FEEDING.

DISINFECTANTS AND METHODS OF DISINFECTION EMPLOYED IN THE HOUSE AND SICK-ROOM.

Disinfection and Disinfectants.—Sunlight, pure air and cleanliness are always very important agents in maintaining health and in protecting the body against many forms of illness. When, however, it becomes necessary to guard against such special dangers as infectious material from communicable diseases the additional protection of disinfection should be considered. Practical disinfection never affords complete protection; and perfect cleanliness is better, even in the presence of contagious disease, than filth with attempted disinfection. If it is possible to disinfect thoroughly the discharges of patients, aërial disinfection is generally unnecessary.

In order that as few articles as possible shall be exposed to the pathogenic germs in the discharges of patients, it is important, when conditions allow, that all articles not necessary for the care and comfort of the sick person, especially upholstered furniture, carpets and curtains, should be removed from the room before placing the sick person in it.

Agents for Cleansing and Disinfection.—Too much emphasis cannot be placed upon the importance of cleanliness of both person and dwelling in protecting the body from all kinds of infectious disease. Personal cleanliness should be attained by frequent cleansing of the hands and body, replacing fabrics infected by expectoration, bowel discharges, etc. By these means most of the pathogenic bacteria are removed before they have caused infection.

Cleanliness in dwellings, and in all places where men go, may, under ordinary circumstances, be well maintained by the use of the two following solutions:

1. **Soapsuds Solution.**—For simple cleansing, or for cleansing after the method of disinfection by chemicals described below, one ounce of common washing soda should be added to twelve quarts of hot soapsuds (soft soap and water).

2. **Strong Soda Solution.**—This is a stronger and more effective cleansing solution and also a fairly efficient disinfectant. It is made by dissolving $\frac{1}{2}$ pound of common washing soda in 3 gallons of hot water. The solution thus obtained should be applied by scrubbing with a hard brush.

When it becomes necessary to destroy completely the causative organism of a communicable disease, in order to prevent its spread, these measures for simple cleanliness are not sufficiently effective and more powerful agents, commonly known as disinfectants, must be employed. The following are some of the most reliable ones:

1. **Heat.**—Complete destruction by fire is an absolutely safe method of disposing of infected articles of small value. Complete sterilization can be attained also by continued high temperature not as great as that of fire. Boiling or steaming in a closed vessel for ten minutes will allow of penetration and will kill all disease germs except spores.

2. **Carbolic Acid Solution.**—Dissolve six ounces of carbolic acid in one gallon of hot water (200 grams in 4000 c.c.). This makes approximately a 5 per cent. solution of carbolic acid, which, for most purposes, may be diluted with an equal quantity of water. The commercial "soluble crude carbolic acid" which is cheaper and twice as effective as the carbolic acid, can be used for privies and drains.[1] It makes a white emulsion owing to incomplete solution. Care must be taken that the pure acid does not come in contact with the skin.

3. **Bichloride Solution** (bichloride of mercury or corrosive sublimate).—Dissolve sixty grains of pulverized corrosive sublimate and two tablespoonfuls of common salt in one gallon of hot water. This solution, which is approximately 1 to 1000, must be kept in glass, earthen, or wooden vessels (not in metal containers). For safety it is well to color the solution.

The carbolic and bichloride solutions are very poisonous when taken by mouth, but are comparatively harmless when used externally.

4. **Milk of Lime.**—This mixture is made by adding 1 quart of dry, freshly slaked lime to 4 or 5 quarts of water. (Lime is slaked by pouring a small quantity of water on a lump of quicklime. The lime becomes hot, crumbles, and as the slaking is completed, turns to a white powder. The powder is used to make milk of lime. Air-slaked lime (the carbonate) has no value as a disinfectant.)

5. **Dry Chlorinated Lime, "Chloride of Lime."**—This must be fresh and kept in closed vessels or packages. It should have the strong, pungent odor of chlorine.

6. **Formalin** (a watery solution containing 40 per cent. of formaldehyde).—Add 1 part of formalin to 10 of water. This equals in value the 5 per cent. carbolic acid solution.

7. **Creolin, Tricresol and Lysol.**—These preparations are about three times as powerful as pure carbolic acid.

The proprietary disinfectants, which are so often widely advertised and whose composition is kept secret, are relatively expensive and often unreliable and inefficient. It is important to remember that substances

[1] The cost of the pure carbolic acid solution is much greater than that of most of the other solutions, but except for the disinfection of woodwork, and for the skin, which in some persons it irritates, it is generally much preferred by those not thoroughly familiar with disinfectants, as it does not deteriorate, and is rather more uniform in its action than some of the other disinfectants.

which destroy or disguise bad odors are not necessarily disinfectants and that there are very few disinfectants that are not poisonous when taken internally. Their value should be stated in the circular in comparison with pure carbolic acid, so that their strength may be known.

Methods of Disinfection in Infectious and Contagious Diseases.—The diseases most commonly guarded against, outside of surgery, by disinfection are scarlet fever, measles, diphtheria, tuberculosis, smallpox and typhoid fever.

1. **Hands and Person.**—Dilute the 5 per cent. carbolic solution with an equal amount of water or use the 1 to 1000 bichloride solution without dilution. Hands soiled in caring for persons suffering from contagious diseases, or soiled portions of the patient's body, should be immediately and thoroughly soaked with one of these solutions and then washed with soap and water, and finally immersed again in the solution. The nails should always be kept clean. Before eating, the hands should be first washed in one of the above solutions, and then thoroughly scrubbed with soap and water by means of a brush.

Care should be taken to allow the bichloride solution to act without contact with soap as the two form a nearly inert compound.

2. **Soiled Clothing, Towels, Napkins, Bedding, etc.,** when removed from the patient should be immediately immersed in the 2.5 per cent. carbolic solution kept in the sick-room, and soaked for one or more hours. Articles which cannot be washed should be thoroughly exposed to formaldehyde gas, as noted later. This is not necessary after exposure to measles.

3. **Food and Drink.**—Thoroughly cooked food and drinks that have been boiled are free from disease germs. Food and drinks, after cooking or boiling, if not immediately used, should be placed when cool in clean dishes or vessels and covered. In the presence of an epidemic of cholera or typhoid fever, water for drinking, cooking, washing dishes, etc., should be boiled before using and milk also should be pasteurized or boiled; likewise raw fruit and uncooked vegetables should not be eaten.

4. **Discharges of all kinds from the Mouth, Nose, Bladder and Bowels** of patients suffering from contagious diseases should be received into glass, metal, or earthen vessels containing the carbolic solution, or milk of lime, or they should be removed on pieces of cloth, which are immediately immersed in one of these solutions or boiled or destroyed by fire. Special care should be observed to disinfect at once the vomited matter and the intestinal discharges from cholera patients. In typhoid fever the urine and the intestinal discharges, and in diphtheria, measles and scarlet fever the discharges from the throat and nose all carry infection and should be treated in the same manner. The volume of the solution used to disinfect discharges should be at least twice as great as that of the discharge, and should completely mix with it and cover it. After standing for an hour or more the discharges with the exception of the feces may be thrown into the water-closet.

Masses of feces are extremely difficult to disinfect except on the surface, for it takes disinfectants such as the carbolic acid solution

some twelve hours to penetrate to their interior. If fecal masses are to be thrown into places where the disinfectant solution covering them will be washed off, it will be necessary to be certain that the disinfectant has previously penetrated to all portions and destroyed the disease germs. This can be brought about by stirring them with the disinfectant and allowing the mixture to stand for one hour, or by washing them into a pot holding soda solution which is already at the boiling temperature, or later will be brought to it.

5. **Sputum from Consumptives.**—In all cases the sputum should be disinfected when discharged. It should be received in covered cups containing the carbolic or milk-of-lime solution. Handkerchiefs soiled by it should be soaked in the carbolic solution and then boiled. Where the rules of cleanliness have not been carried out in rooms occupied by consumptive patients, the dust from walls, mouldings, pictures, etc., will frequently be found to contain the germs and will produce tuberculosis when inoculated into animals; such rooms, therefore, should be thoroughly renovated or disinfected before they are occupied again. If the sputum of all consumptive patients were destroyed as soon as discharged, a large proportion of the cases of the disease would be prevented.

6. **Closets, Sinks, etc.**—Infected discharges should never be disposed of until they have been rendered innocuous by thorough disinfection. They may then be emptied into the closet. As an added precaution, after the pan is flushed, a quart of carbolic solution should be poured into the pan and allowed to remain there. Sinks should be flushed with one of these solutions at least once daily.

7. **Dishes, Knives, Forks, Spoons, etc.**, used by a patient suffering from a communicable disease should, as a rule, be kept for his exclusive use. They should be washed first in the carbolic solution, then in boiling hot soapsuds, and finally rinsed in hot water. The remains of the patient's meals may be burned or thrown into a vessel containing the carbolic solution or milk of lime, and allowed to stand for one hour before being thrown away.

8. **Rooms and Their Contents.**—When the patient is freed from isolation probably the disease germs have already died, but a few may have survived. The danger from infection is much greater when cases are removed during the acute illness. For disinfecting rooms careful fumigation with formaldehyde gas should be employed unless vermin are to be killed, when sulphur fumes should be substituted. Carpets, curtains and upholstered furniture which have been soiled by discharges, or which have been exposed to infection during the illness, may be removed for disinfection to chambers where they can be exposed to formaldehyde gas and moderate warmth for twelve to twenty-four hours, or to steam. Some carpets, such as many Wiltons, are discolored by moist steam. These must be put in the formaldehyde chamber. This difficult procedure may be safely omitted, for even if a few germs remain alive in the depth of the fabrics, they probably cannot escape. Wood-work, floors and plain furniture should be washed thoroughly with soapsuds

and bichloride solutions. After the disinfection is finished it is well to remove the dried bichloride of mercury from the walls.

9. **Rags, Cloths and Articles of Small Value,** which have been soiled by discharges or infected in other ways, should be boiled or burned.

10. **In Case of Death** the body should be wrapped completely in several thicknesses of cloth wrung out of the carbolic or bichloride solution, and when possible placed in a hermetically sealed coffin.

It is important to remember that *an abundance of fresh air, sunlight and absolute cleanliness* not only help protect the attendants from infection and aid in the recovery of the sick, but directly eliminate the bacteria which cause disease.

Methods of Cleanliness and Disinfection to Prevent the Occurrence of Illness.—1. **Water-closet Bowls and all Receptacles for Human Excrement** should be kept perfectly clean by frequent flushing with a large quantity of water, and should be disinfected with the crude carbolic or other efficient solutions as often as necessary. The wood-work around and beneath them should be scrubbed frequently with the hot soapsuds solution.

2. **Cesspools and Privy Vaults.**—A liberal amount of milk of lime or chloride of lime should be thrown into these daily, and their contents should be removed frequently.

3. **The Wood-work in School-houses** should be wiped daily with warm soapsuds. This refers to floors, doors, door-handles and all wood-work touched by the scholars' hands.

4. **Spittoons in all Public Places** should be emptied daily and washed with the hot soapsuds solution, after which a small quantity of the carbolic solution or milk of lime should be allowed to remain and so mix with the expectoration, thus preventing the conveyance of infection by flies.

5. **Cars, Ferry-boats and Public Conveyances.**—The floors, door-handles, railings, and all parts touched by the hands of passengers should be washed frequently with the hot soapsuds solution. Slat-mats from cars, etc., should be cleaned by scrubbing with a stiff brush in the hot soapsuds solution.

Telephone mouth-pieces also should be cleansed frequently.

The Practical Employment of Formaldehyde Gas in the Surface Disinfection of Rooms and the Disinfection of Goods which would be Injured by Heat.—Formaldehyde gas has come into such general use, and is for many purposes so valuable, that the description of methods employed to generate and use it will be given in detail.

If we consider now the practical application of formaldehyde gas for purposes of disinfection we find that its destructive action on micro-organisms depends upon a number of factors, the chief of which are its concentration in the surrounding atmosphere, the length of the contact, the temperature, the accompanying moisture, and the nature of the organism. It is not an insecticide like sulphur dioxide.

Concentration.—The concentration of gas in the surrounding atmosphere necessary to kill microörganisms varies with each species (some

resist chemical agents much more than others) and also with the freedom of access of the gas to the bacteria.

For purely surface disinfection, when the common pathogenic bacteria are to be destroyed, there will be required, according to the method used, ten to twelve ounces of formalin of full strength, or its equivalent, to 1000 cubic feet of air space.

The gas penetrates through fabrics with difficulty, and to pass through heavy goods the concentration of the gas must be doubled and the temperature must be raised to 45° C. or over.

Although formaldehyde gas does destroy bacteria in the presence of moderate atmospheric moisture, it acts much more powerfully and certainly when additional moisture is present, and best when present up to the point of saturation. The actual spraying with water of walls and goods to be disinfected is even more efficacious.

Temperature.—A fairly high temperature—but one still below that which would injure delicate fabrics—increases not only the activity of formaldehyde gas, but also its penetrative power. For heavy goods this is essential. The production of a partial vacuum in the chambers before the introduction of the formaldehyde gas still further assists its penetration.

Length of Exposure.—This depends upon the nature of the disease for which it is carried out and varies with the penetration required, the concentration of the gas used, the amount of moisture in the air, the temperature of the air, and the size and shape of the room. For surface disinfection in rooms, when as much as 12 ounces of formalin are used for each 1000 cubic feet, five hours' exposure is amply sufficient, most bacteria being killed within the first thirty minutes. For the destruction of microörganisms protected by even a thin covering, a double amount of formalin and double time of exposure should be allowed, and even then the killing of many species of non-spore-bearing bacteria cannot be counted upon in ordinary rooms. When absolutely complete disinfection is demanded and penetration of the gas is required, the goods must be placed in chambers where moderate heat can be added and all leakage of gas prevented.

In order to insure complete sterilization articles should be so placed as to allow free circulation of the gas around them; bedding, clothing, etc., should be either spread out on perforated wire shelves or loosely suspended in the chamber. The aid of a partial vacuum facilitates the operation. Upholstered furniture and articles requiring much space should be placed in a large chamber, or, better, in a room which can be heated to the required temperature.

The most delicate fabrics, furs, leather, and other articles, which are injured by steam, hot air at 230° F., or other disinfectants, are unaffected by formaldehyde.

Disinfection of Books.—Books may be satisfactorily disinfected by means of formaldehyde gas in a special room, or in the ordinary steam chamber, as above described, and under the same conditions of temperature, volume of gas, and time of exposure. The books should

be arranged to stand as widely open as possible upon perforated wire shelves, set about 1½ feet apart. A chamber having a capacity of 200 to 250 cubic feet would thus afford accommodation for about 100 books at a time.

Books, with the exception of their surfaces, cannot be disinfected satisfactorily by formaldehyde gas in the bookcases of houses or libraries, or anywhere except in special chambers constructed for the purpose, because the conditions required for their thorough disinfection cannot otherwise be complied with.

Disinfection of Carriages, Etc.—Carriages, ambulances, cars, etc., can be disinfected easily by having built a small, tight building, in which they are enclosed and surrounded with formaldehyde gas. Such a building is used for disinfecting ambulances in New York City. With the apparatus there employed a larger amount of formalin is rapidly vaporized, and superficial disinfection is completed in sixty minutes.

Methods of Generating Formaldehyde Gas.—Various forms of apparatus can properly be employed to liberate formaldehyde gas for purposes of disinfection. There are two essentials to any good method—namely, that the formaldehyde gas shall be given off quickly, and that there shall be no great loss by deterioration of the formalin.

Formalin by Boiling and Passing the Vapor through a Superheated Coil or Chamber.—This system consists in heating the ordinary commercial formalin to a high temperature in an incandescent copper coil or chamber, and allowing the vapors to pass off freely. It is claimed that this method supplies the degree of heat necessary to break up the polymerized products as they form and thus prevent a loss of formaldehyde. Furthermore, the intense heat in the copper tube converts part of the methyl alcohol present in commercial formalin into formaldehyde gas, by incomplete oxidation, thereby increasing the amount of formaldehyde set free. (Fig. 215.)

Trioxymethylene or Paraform.—This system consists in heating the solid polymer of formaldehyde (trioxymethylene) and thus changing it to the gaseous form.

There are several methods for doing this. They are somewhat expensive but efficient and are very generally used. The formaldehyde gas must be protected from burning.

Formalin to which Glycerin has been Added.—To the formalin is added 10 per cent. of glycerin, and the mixture is simply boiled in a suitable copper vessel, the steam and formaldehyde gas passing off by a tube. This is a very serviceable apparatus. When an attempt is made to vaporize the formalin too rapidly part of it bubbles over in liquid form.

With 50 per cent. more of formalin than that used in the high temperature autoclave and heated tube or chamber methods, the results seem to be equally good. The apparatus is very easy to use, and is not liable to get out of order.

Similar forms of apparatus are also employed, when, instead of glycerin, the formalin is mixed with an equal quantity of water. The water is for the purpose of giving additional moisture to the air, and, at the same time, like the glycerin, to prevent the change of formaldehyde into inert substances.

Formalin on Sheets.—A very simple method is to stretch a number of lines across the room, spread a sufficient number of sheets over the lines and drench

with formalin, using 16 ounces per 1000 cubic feet. If the room is tightly sealed very fair surface disinfection will take place in two hours.

Rapid Generation of Formaldehyde Gas for Large Chambers by the Method of Dr. R. J. Wilson.—The generator (Fig. 216) is made of ordinary iron steam pipe and can be manufactured in any pipe-cutting establishment in a very few hours. It consists of an outer steam jacket of six-inch pipe, two feet long and capped at both ends. Through the upper cap there is a four-inch opening with a thread, through which projects an inner chamber for formalin. This chamber consists of a four-inch pipe, twenty-two inches long, capped at the upper end and welded or capped at the lower end. The upper end of this pipe is so threaded as to permit of its being screwed through the cap of the steam jacket before that cap is screwed on. The cap of the formalin chamber is fitted on the same thread that passes through the cap of the steam jacket. The intake for steam is near the top of the steam jacket, through a half-inch pipe, and the steam is controlled by a globe valve. The outlet for steam or drip is through a half-inch pipe from the bottom cap of the chamber and is also controlled by a globe valve. The intake for formalin is through the upper cap of the formalin chamber through a half-inch pipe controlled by a globe valve. The outlet for formaldehyde is a half-inch pipe through the upper cap of the formalin chamber.

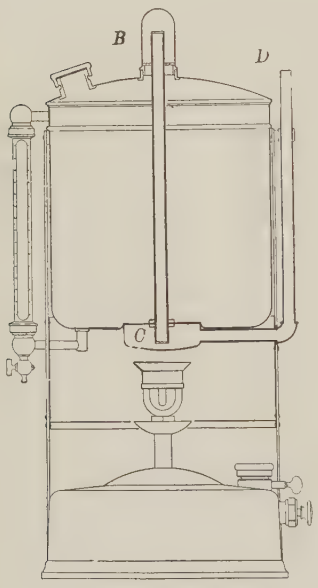


Fig. 215.—Formaldehyde apparatus.

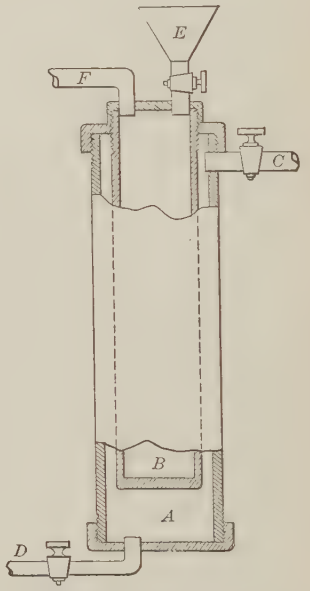


Fig. 216.—Wilson's formaldehyde generator: *A*, steam chamber; *B*, formalin chamber; *C*, steam supply; *D*, drip; *E*, inlet for formalin; *F*, outlet for formaldehyde.

Method for Testing Efficacy of Room Disinfection.—The following method, which was adopted by us after investigations in the Department of Health Laboratories and modification by Dr. M. C. Schroder, seemed to be the best means of determining the efficacy of room disinfection and has been used in the Health Department. It

consists in the use of infected threads and the main points of the plan are as follows:

No. 36 cotton is cut into inch lengths, placed in a Petri dish, and covered with a forty-eight-hour broth culture of B. pyocyaneus.

They are left for two or three minutes or until they are thoroughly saturated, then removed to filter-paper in another covered Petri dish and left to dry. When dry they are placed in tissue-paper envelopes, which are stamped with all necessary data. Each envelope is dated and sealed and sent to the disinfector who places it in the room which is to be disinfected.

The driver who calls for the bedding takes up the tests, placing them in a manila envelope and entering them upon his card. The envelopes are then returned to the laboratory where the tests and receipt card are compared and any discrepancy noted.

The test envelopes are then stamped with date of receipt, and the threads are removed and placed in a modified Ayer's medium, which is a synthetic medium made up as follows:

Asparagin	4
Neutral sodium phosphate	2
Sodium lactate	6
Sodium chlorate	5
Water	1000

Add enough NaOH to render the medium alkaline to litmus. This culture medium may be depended upon to give bright green color reaction in twenty-four to forty-eight hours.

The tubes are incubated for forty-eight hours and the color reaction noted and entered upon test envelope.

At the end of the week a bacteriologist's report is compiled which shows at a glance the work of each disinfector, the number of cases of each disease for which disinfection was performed, the number of successful disinfections, the number of tests lost, etc.

Sulphur Dioxide in House Disinfection.—Four pounds of sulphur should be burned for every 1000 cubic feet. The sulphur should be broken into small pieces and put into a pan sufficiently large so that the melted sulphur cannot overflow. This pan is placed in a much larger pan holding a little water. Upon the broken sulphur is poured 3 or 4 ounces of alcohol and the whole lighted by a match. The alcohol is not only for the purpose of aiding the sulphur to ignite, but also to add moisture to the air. Before beginning disinfection the cracks of the room should be carefully pasted up and the door, after closing, also sealed. An exposure of eight to twelve hours should be given.

Sulphur fumigation carried out as above indicated is not as efficient as formaldehyde fumigation but suffices for surface disinfection for diphtheria and the exanthemata. All heavy goods should be removed for steam disinfection if there is any possibility of the infection having penetrated beneath their surface. If there is no place for steam disinfection they should be thoroughly exposed to fumigation and then to the air and sunlight. In many cases when cleanliness has been observed,

surface disinfection of halls, bedding and furniture may be all that will be required.

There is always a very slight possibility of a deeper penetration of infection than that believed to have occurred; it is therefore better to be more rather than less thorough than is considered necessary.

Sulphur dioxide without the addition of moisture has, as already stated under the consideration of disinfectants, very little germicidal action upon dry bacteria.

Advantages of Formaldehyde Gas over Sulphur Dioxide for Disinfection of Dwellings.—Formaldehyde gas is superior to sulphur dioxide as a disinfectant for dwellings, first, because it is more efficient in its action, second, because it is less injurious in its effects on household goods, and third, because when necessary it can be supplied easily from a generator placed outside of the room and watched by an attendant, thus avoiding all danger of fire.

Apart from the cost of the apparatus and the greater time involved, formaldehyde gas, generated from commercial formalin, is not much more expensive than sulphur dioxide—viz., twelve to twenty cents per 1000 cubic feet against ten cents with sulphur. Therefore, we believe that formaldehyde gas is the best disinfectant at present known for the surface disinfection of infected dwellings. For heavy goods it is far inferior in penetrative power to steam, but for the disinfection of fine wearing apparel, furs, leather, upholstery, books, and the like, which are injured by great heat, it is, when properly employed, more suitable than any other disinfectant now in use.

Hydrocyanic Acid Gas.[1]—This gas has no germicidal power although it is a powerful poison for all forms of animal life. It is used extensively for killing insects and rats, particularly on ships. Its use is accompanied by considerable danger to man and should be used only by persons familiar with its properties and methods of use. The gas may be generated by several methods, but one of the best consists of mixing hot sulphuric acid, water and sodium or potassium cyanide in large lumps. In ships the following proportions are used for 1000 cubic feet of space:

3¾ ounces of sodium cyanide
5 fluid ounces (9 ounces by weight) H_2SO_4
7½ ounces of water

Larger quantities are required for houses, mills, barns, etc., where there are open cracks. The vessel for generating the gas should be of wood or stone. Large enough vessels must be used to prevent frothing over. Water is put in first, then the acid is added slowly with mixing, followed by the cyanide. The operator must escape immediately or he will be killed by the gas. An expert should determine when it is safe to return to the room. Lachrymatory gases such as CNCl are sometimes added in order to give warning of the presence of the HCN.

Recent experiments[2] have shown that within tightly sealed rooms the natural diffusion of HCN is primarily upward, so that it is important

[1] Disinfectants, Public Health Bulletin No. 42, 1917.
[2] Public Health Reports, 1928, vol. **43**, No. 41.

that the gas be generated at low levels of the compartment. Conclusions from these experiments are as follows: No ordinary concentration and no ordinary exposure time will insure the infiltration of the gas into small openings used by rats. Therefore it is essential to either open up or eliminate these small spaces. A ship not properly prepared will nullify the good effects of careful gassing.

Liquid hydrocyanic gas in cylinders may be used also. It is, however, explosive and undergoes decomposition.

Persons engaged in using HCN should wear masks.

Public Steam Disinfecting Chambers.—These should be of sufficient size to receive all necessary goods. They may be either cylindrical or rectangular in shape, and are provided with steam-tight doors opening at either end, so that the goods put in at one door may be removed at the other. Large doors are handled by convenient cranes and drawn tight by drop-forged steel eye-bolts swinging in and out of slots in the door frames. The chambers should be able to withstand a steam pressure of at least $\frac{1}{2}$ an atmosphere, and should be constructed with an inside jacket, either in the form of an inner and outer shell or of a coil of pipes. This jacket is filled with steam during the entire operation, and is so used as to heat the goods in the disinfecting chamber to about 220° F. before allowing the steam to pass in. After this preliminary heating the steam does not condense on coming in contact with the goods. It is an advantage to displace the air in the chamber before throwing in the steam, as hot air has far less germicidal value than steam of the same temperature. To do this a vacuum pump is attached to the piping, whereby a vacuum of 15 inches can be obtained in the chamber. The steam should be thrown into the chamber in large amount, both above and below the goods, and the excess should escape through an opening in the bottom of the chamber, so as more readily to carry off with it any air still remaining. The live steam in the chamber should be under a pressure of 2 or 3 pounds to increase its action.

To disinfect the goods we place them in the chamber, close tight the doors, and turn the steam into the jacket. After about ten minutes, when the goods have become heated, a vacuum of ten to fifteen inches is produced, and then the live steam is thrown in for twenty minutes. The steam is now turned off, a vacuum is again formed, and the chamber again superheated. The goods are now thoroughly disinfected and dry. In order to test the thoroughness of any disinfection, or any new chamber, maximum thermometers are placed, some free in the chamber and others surrounded by the heaviest goods. It will be found that, even under a pressure of three pounds, live steam will require ten minutes to penetrate heavy materials.

Practical Points on Heat Disinfection.—In the practical application of steam for disinfecting purposes it must be remembered that while moist steam under pressure is more effective than streaming steam, it is scarcely necessary to give it the preference, in view of the fact that most known pathogenic bacteria produce no spores, and the few that do, produce spores that are quickly destroyed by the temperature of

boiling water; also that "superheated" steam is less effective than moist steam. When steam confined in pipes is "superheated" after its generation it has about the same germicidal power as hot, dry air at the same temperature. Esmarch found that anthrax spores were killed in streaming steam in four minutes, but were not killed in the same time by superheated steam at a temperature of 114° C. It should be remembered also that dry heat has but little penetrating power, and that even steam requires time to pass through heavy goods. Koch and Wolffhügel found that registering thermometers placed in the interior of folded blankets and of other large packages in a dry hot-air oven at 133° C. and over did not show, after three hours' exposure, a temperature capable of killing bacteria. We have put a piece of ice in the middle of several mattresses and recovered it after exposing the goods to an atmosphere of live steam for ten minutes.

Gauze.—Gowns, towels, pail pads, caps, masks are autoclaved at 15 pounds for forty minutes. They are placed in a perforated cylinder or wrapped in clean towels before putting in the sterilizer, and opened only at the operation.

Ligatures—Catgut.—Dehydrate slowly by heating for twenty-four hours at 240° F. dry heat. Boil in albolene or kumol for two hours, pour off albolene and place in 95 per cent. alcohol. They are often put in sealed glass tubes, which are boiled under pressure. These remain sterile indefinitely. The alcohol does not injure the catgut. If desired, the catgut can be washed in ether and then soaked a short time in bichloride before heating in alcohol. Böckman, of St. Paul, suggested wrapping the separate strands of catgut in paraffin paper and then heating for three hours at 140° C. This procedure prevents the drying out of the moisture and fat from the catgut, so that it remains unshrivelled and flexible after its exposure. Darling, of Boston, tested this method and found it satisfactory. Dry formaldehyde gas does not penetrate sufficiently, and is not reliable. Silver wire, silk, silkworm gut, rubber tubing and catheters are boiled in the same way as are the instruments.

Hand-brushes.—These should be boiled in soda solution for ten minutes.

The Skin of the Patient.—It is impossible to sterilize absolutely the deeper portions of the skin, but sufficient bacteria can be removed to render infection rare. The skin is washed thoroughly with warm green soap solution, then with alcohol, and finally with 1 to 1000 bichloride. A compress wet with a 25 per cent. solution of green soap is now placed on, covered with rubber tissue, and left for three to twelve hours; after its removal the skin is washed with ether, alcohol, and bichloride solution, and then covered with a gauze compress previously moistened with a 1 to 1000 bichloride of mercury solution. At the operation the skin is again scrubbed with green soap solution followed by ether, alcohol, and then with the bichloride of mercury solution. In some places the bichloride compress is replaced one hour before the operation by a pad wet in 10 per cent. solution of formalin.

Tincture iodine, 7.5 per cent for adults, 3 per cent for children, may be painted on the skin when the above methods cannot be used. It is left on until it dries and then washed off with 75 per cent. alcohol.

The Hands.—Fürbinger's method, slightly modified, is now much used, and gives good results. The hands are washed in hot soap and water for five minutes, using the nail-brush. They are then soaked in 85 per cent. alcohol for one minute and scrubbed with a sterile brush. They are finally soaked in a 1 to 1000 bichloride of mercury solution for two minutes. The alcohol and bichloride of mercury are sometimes combined and used together. Another method which gives good results is as follows: Skin of operator is scrubbed for five minutes with green soap and brush, then washed in chorinated lime and carbonate of soda in proportions to make a good lather, washed off in sterile water and then scrubbed with a brush in warm bichloride solution 1 to 1000.

Owing to the risk of leaving untouched bacteria under the nails and in cracks of the skin, sterilized rubber gloves are in general use in operations. New gloves must be scrubbed with brush, soap and water and boiled before sterilizing to remove dressing in rubber. After operation they are washed with cold water to remove blood. When washed and boiled as above they are put up in two covers. They are sterilized either by boiling for five minutes and put on wet, or put in autoclave at 5 pounds' pressure for one hour, or 15 pounds' pressure for fifteen minutes. Some surgeons prefer sterilized cotton gloves frequently changed. The gloves can be sterilized by steam.

Mucous Membranes.—Here absolute sterilization cannot be achieved without serious injury to the tissues. The mouth and throat are cleansed by a solution consisting of equal parts of peroxide of hydrogen and lime water. In the nostrils it is better to employ the milder solutions, such as diluted Dobell's or Listerine. These are also used in the mouth instead of the peroxide. Wadsworth[1] urges the use of preparations containing about 30 per cent. of alcohol as being very efficient. Many disinfectant solutions are swabbed or sprayed on the mucous membrane.

The vagina is swabbed out thoroughly with sterile warm soap and water, and then irrigated with a 2 per cent. carbolic acid or a 1 to 1000 bichloride of mercury solution.

Disinfection of Wounds.—The great efficiency of chlorine and some of its less stable compounds as purifying, deodorizing and bleaching agents has for a long time been known. Its action was atrributed to its strong indirect oxidizing influence. Chlorine gas and calcium hypochlorite, "bleaching powder" or "chloride of lime," were recognized as among the most potent, though destructive disinfectants. Potassium and sodium hypochlorite were also employed as disinfectants and to some extent as antiseptics, though the latter use was restricted by the irritating action of these substances.

It was not until the recent war in Europe created a demand for more

[1] Jour. Inf. Dis., 1906, p. 779.

efficient antiseptics than those in general surgical use that recourse was made to chlorine compounds in which the chlorine existed in a combination from which it could be liberated readily and in active form according to requirements.

The researches of Dakin,[1] at first associated with Carrel in France demonstrated that sodium hypochlorite in strictly neutral solution and not exceeding 0.5 per cent. in concentration, could be used freely in surgical practice without occasioning discomfort. His studies on this antiseptic showed that when it acted on protein substances it conferred antiseptic properties on them and that the chlorine presumably became linked to a nitrogen of one or more of the amino-acids composing the protein. This led to the inference, which was verified by experiment, that soluble substances possessing a nitrogen-chlorine linkage would be likely to have antiseptic properties. The group of substances called chloramines, which have this linkage, contain many preparations which are soluble and only slightly, if at all, toxic. The cheapest of these, because it can be manufactured from a waste substance obtained in the production of saccharin, is called Dichloramin-T. This substance is obtained in white crystals readily soluble in water and it is singularly stable both dry and when in solution. It parts readily with chlorine when brought into contact with easily oxidizable substances, and is a powerful antiseptic. Its penetration when applied to the tissues is unusually great. As it is decomposed in exerting its action, it should be frequently renewed, but its non-toxic and unirritating characters permit its free and abundant use.

The limits of the use of gentian violet, mercurochrome, acraflavine, chinosol and others are being studied. It is believed that for certain infections special antiseptics should be selected. In the latter part of the war fresh wounds were usually closed without the use of antiseptics when the injured tissues could be first removed.

Hypodermic and Other Syringes.—These are either boiled or sterilized by drawing boiling water up into them a number of times and then a 5 per cent. solution of carbolic acid, the acid after three minutes to be rinsed out with boiling water. If cold water is used the carbolic solution should remain in the barrel for ten minutes. Great care should be taken to wash out all possible organic matter before boiling or using the carbolic acid. Syringes made entirely of glass or of glass and asbestos can be boiled in soda solution.

GENERAL CONCLUSIONS ON DISINFECTION.

The previous pages have shown that it is comparatively easy to destroy microbes by germicides or heat when access to them is attainable. Foods, instruments, clothing, bedding, the excreta, the surface of the body, etc., can be readily disinfected, but when we try to disinfect the mucous membranes of the living person we fail.

[1] British Med. Jour., 1915, 2, 318; Ibid., January 29, 1916, p. 160.

The Importance of Disinfection of Surroundings after Recovery or Death from Infectious Diseases.—Year by year knowledge is accumulating which indicates that nearly all cases of spread of infection are due to the immediate transfer from a living carrier to the person who contracted the disease or to the indirect transfer through the contamination of food or water. The carrier may be diseased or may simply harbor the germs. If cleanliness is maintained throughout the disease there is, as a rule, little need of specific disinfection after recovery.

The Danger from Infected Corpse.—The pathogenic bacteria remaining in the bodies of those who have died from communicable diseases are not dangerous if the body is so treated and covered that infected fluids can not escape and no one can touch an infected portion.

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